University of Alberta

Characterization of Ligand Interaction at the γ-Aminobutyric Acid Type A Receptor

by

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ABSTRACT

Ligands of different structural classes and ranging the full spectrum of efficacies interact with the γ-aminobutyric acid type A ($GABA_A$) receptor. Characterization of these interactions is important to enhancing knowledge of the structure-function relationships of this ligand-gated ion channel and improving design of pharmacological agents that target the receptor with greater specificity. This thesis examines various ligand interactions at the benzodiazepine binding site or agonist binding sites of recombinant wild type and mutant $GABA_A$ receptor subtypes.

The affinities of amentoflavone, a biflavonoid, and 5′-bromo-2′-hydroxy-6-methylflavone, a synthetic flavonoid derivative, for the benzodiazepine binding site were investigated using wild type and mutant $GABA_A$ receptors. Functional effects of these compounds were also examined. These studies revealed a complex mechanism of flavonoid interaction at $GABA_A$ receptors. The pattern of binding at the benzodiazepine site of wild type $GABA_A$ receptor subtypes expressing different α subunits was observed to resemble that of the classical benzodiazepine agonist diazepam, and the importance of the histidine 101 residue (rat α_1 subunit) was indicated. In addition, results suggest that functional modulation of the receptor by amentoflavone occurs by a mechanism independent of the benzodiazepine and loreclezole sites.

Affinity and efficacy changes for the β-carboline negative modulator, ethyl-β-carboline-3-carboxylate (β-CCE) were compared using the histidine 101 mutant $GABA_A$ receptors. Results revealed that changes in efficacy do not parallel changes in apparent affinity; however, a comparison of these findings with those of previous studies examining the imidazobenzodiazepine antagonist Ro15-1788 and inverse agonist Ro15-

4513 suggest possible benzodiazepine site recognition properties based on the efficacy of a ligand.

Agonist responses of GABA, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), and piperidine-4-sulfonic acid (P4S) were studied at α_4-, α_6-, and chimeric α_6/α_4 subunit-containing $GABA_A$ receptor subtypes to determine whether a specific domain could be identified which confers differential sensitivity to the subunits. Results of this study did not determine differential sensitivity of α_4- versus α_6-containing receptors to GABA site agonists. However further research comparing these two receptor subtypes, where the δ subunit is incorporated as opposed to $\gamma_{2,}$ is indicated in light of recent research findings demonstrating the effect of extrasynaptic subunit composition on agonist potency and efficacy.

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To my daughters,

Jasmyn and Azalea
Hansen

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LIST OF ABBREVIATIONS

AChBP	acetylcholine binding protein
βCCE	ethyl β-carboline-3-carboxylate
DNA	deoxyribonucleic acid
DI	diazepam-insensitive
DS	diazepam-sensitive
DZ	diazepam
FZ	flunitrazepam
GABA	gamma-aminobutyric acid
GABA$_A$	gamma-aminobutyric acid type A
GAD	glutamate decarboxylase
5HT	5-hydroxytryptamine
5HT$_3$	5-hydroxytryptamine type 3
4-PIOL	5-(4-piperidyl)-3-isoxazolol
P4S	piperidine-4-sulfonic acid
RNA	ribonucleic acid
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol
TM	transmembrane

CHAPTER 1

Introduction

The balance between neuronal inhibition and excitation underlies the basic functioning of the brain (Whiting, 2003). γ-Aminobutyric acid, or GABA, is the major inhibitory neurotransmitter in the mammalian central nervous system, estimated to have actions at 20-50% of synapses (Sieghart, 1995; Barnard et al., 1998; Cooper et al., 2003; Bateson, 2004). GABA is synthesized in GABAergic nerve terminals from its precursor, glutamate, by the enzyme glutamate decarboxylase (GAD), accumulated into synaptic vesicles, and released into the synaptic cleft upon depolarization (Cooper et al., 2003). The synaptic actions of GABA are mediated through GABA receptors including the $GABA_A$ receptor, a member of the superfamily of ligand-gated ion channels, the $GABA_B$ receptor, a G-protein coupled receptor, and the $GABA_C$ receptor, a $GABA_A$ receptor subtype (Sieghart, 1995; Barnard et al., 1998). The $GABA_A$ receptor in particular is believed to play a central role in mediating the fast-inhibitory activity of GABA and, as such, has become the target of numerous clinically important drugs (Cooper et al., 2003; Bateson, 2004). The loss of regulation of either excitation or inhibition in the brain is postulated to be a factor in various neurodegenerative and psychiatric conditions (Whiting, 2003). Research has focused on the development of pharmacological therapies to correct this imbalance as a treatment for conditions such as epilepsy and schizophrenia. Drugs which target the $GABA_A$ receptor have proven clinically useful for the treatment of epilepsy, anxiety, sleep disorders, and alcohol withdrawal, as well as for the generation of anesthesia (Korpi et al., 2002). The $GABA_A$ receptor therefore continues to be a prime target for pharmacological intervention.

1. $GABA_A$ receptor structure

Isolation and partial amino acid sequencing, followed by subsequent cloning, of the first $GABA_A$ receptor subunits led to the determination that this receptor belonged to a superfamily of ligand-gated ion channels (Schofield et al., 1987). This superfamily, which includes the nicotinic acetylcholine receptor, the 5-hydroxytryptamine type 3 ($5HT_3$) receptor and the glycine receptor, is also known as the 'cys-loop family' due to the characteristic cysteine loop found in the N-terminal domain of their subunits (Wafford, 2005). Similar to the other members of the superfamily, the $GABA_A$ receptor is composed of five subunits arranged in a rosette conformation around a central chloride-selective ion channel (Fig. 1-1; Nayeem et al., 1994). The identification of different subunit classes, several isoforms within a subunit class, as well as the existence of splice variants of some subunits (e.g. γ_2 short and γ_2 long) has revealed the extensive heterogeneity of GABA$_A$ receptors (Schofield et al., 1987; Levitan et al., 1988; Whiting et al., 1990). Thus far, 19 subunits have been identified, including 6α, 3β, 3γ, δ, ε, π, θ, and 3ρ subunits belonging to the GABA$_C$ subtype (Barnard et al., 1998; Sieghart et al., 1999; Korpi et al., 2002). The subunits themselves share a basic structural homology: a large extracellular N-terminal domain containing the conserved cysteine residues linked by a disulfide bond, four transmembrane (TM) domains– TMII of which forms the lining of the ion channel pore, a large intracellular loop between TMIII and TMIV where phosphorylation sites are thought to exist, and a small extracellular C-terminal domain (Fig. 1-2; Sieghart, 1995). The subunit composition of the heteropentameric GABA$_A$ receptor determines its pharmacology, and although the number of receptor subtypes that have been shown to exist physiologically is limited compared to the multiple combination possibilities of the subunits identified thus far (McKernan and Whiting, 1996; Bollan et al., 2003b), the broad diversity of GABA$_A$ receptor subtypes continues to be a significant focus of research with regard to specificity of therapeutic targeting. The majority of GABA$_A$ receptor subtypes are suggested to be composed of α, β, and γ subunits with a stoichiometry of 2α:2β:1γ (Sieghart et al., 1999) and the clockwise arrangement of γ-α-β-α-β as seen from the extracellular view (Fig. 1-3; Trudell, 2002).

2. Regional distribution of GABA$_A$ receptors

Regional brain expression patterns showing discrete distributions of specific receptor subunits have also provided clues as to the different functional roles that particular GABA$_A$ receptor subtypes fulfill (Wisden et al., 1992; Pirker et al., 2000; Wafford, 2005). The $\alpha_1\beta_2\gamma_2$ combination is thought to be the most abundant receptor subtype in the mammalian central nervous system (Whiting, 2003). Other GABA$_A$ receptor subtypes display more distinct distributions in the brain. These include α_5-containing receptors which are found primarily in the hippocampus, an area associated with learning and memory (Wisden et al., 1992, Whiting, 2003), α_6-containing receptors highly expressed in the cerebellular granule cells, which are known to be of importance in the execution of motor tasks (Wisden et al., 1992; Korpi et al., 1999; Sieghart et al., 1999), and extrasynaptic GABA$_A$ receptors likely containing α_4, α_5, α_6, and δ subunits, which are thought to play a significant role in mediating tonic inhibition (Mody, 2001; Whiting, 2003).

3. GABA$_A$ receptor pharmacology

The pharmacology of the GABA$_A$ receptor is complex. As stated, the specific combination of subunits determines the properties of a particular GABA$_A$ receptor subtype. The chloride current flowing through the integral ion channel of the receptor is gated by binding of the endogenous agonist GABA (Sieghart, 1995). Muscimol and isoguvacine also seem to act as full GABA$_A$ receptor agonists, as they are able to activate the chloride conductance to a similar extent as GABA. Other compounds such as 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), piperidine-4-sulfonic acid (P4S), and 5-(4-piperidyl)-3-isoxazolol (4-PIOL) seem to exhibit partial agonist activity at major subtypes of the GABA$_A$ receptor. In addition, the GABA-induced chloride current can be allosterically modulated by a number of different classes of compounds. These include the barbiturates, the benzodiazepines, the cyclopyrrolones, ethanol, anesthetics, zinc, steroids, and flavonoids (Sieghart, 1995; Medina et al., 1997; Whiting, 2003; Bateson, 2004). Although research into the mechanisms of action for these compounds continues to characterize their interactions at the GABA$_A$ receptor, the exact binding site(s) and functional effects of some of these compounds remain to be identified and well described. For instance, flavonoid interaction at the GABA$_A$ receptor includes binding at the benzodiazepine binding site, as well as interaction with a second, low affinity site that has yet to be identified (see Chapters 3 and 4). The exact mechanisms of steroid interaction at the GABA$_A$ receptor are also yet to be fully elucidated (Sieghart, 1995). GABA$_A$ receptors are speculated to play a central role in mediating both the short-term and long-term effects of ethanol in the central nervous system (Davies, 2003). The idea of a putative ethanol binding site at the GABA$_A$ receptor has further been substantiated by recent findings describing the competitive inhibition of [^{3}H]Ro15-4513 binding at specific GABA$_A$ receptor subtypes by low ethanol concentrations (Wallner et al., 2006). Despite the need for further research to fully characterize mechanisms of interaction, allosteric modulators have been shown to encompass the full spectrum of efficacy, with positive modulators potentiating GABA-gated chloride current and negative modulators reducing this current, respectively. As well, antagonists will bind to the receptor but have no effect on the chloride current induced by GABA. With respect to inhibitory and excitatory forces in the brain, as the equilibrium potential of chloride is close to the resting potential in most neurons, potentiation of GABA-gated chloride current hyperpolarizes these neurons and decreases the depolarizing effects of an excitatory input (Cooper et al., 2003). This mechanism of depressing excitability has been targeted in the treatment of conditions characterized by excessive excitability, such as epilepsy and anxiety (Whiting, 2003).

4. Benzodiazepine pharmacology

The benzodiazepines are perhaps the best known of all the classes of allosteric modulatory compounds that interact at the $GABA_A$ receptor. Clinically useful benzodiazepines potentiate the GABA-gated chloride current at the $GABA_A$ receptor by increasing the frequency of chloride channel opening (Study and Barker, 1981). Excellent correlation between benzodiazepine affinity for the $GABA_A$ receptor-associated binding site and the clinical potency of these drugs has led to the belief that the important therapeutic actions of the benzodiazepines are primarily exerted via the benzodiazepine binding site of the $GABA_A$ receptor (Sieghart, 1995). In clinical use, the classical 1,4-benzodiazepines, including the compounds diazepam and flunitrazepam, produce sedative-hypnotic, anxiolytic, anticonvulsive, myorelaxant, and amnesic effects. Despite being widely prescribed for the treatment of disorders such as epilepsy, anxiety, and insomnia, this class of drugs is often associated with the development of tolerance, dependence, and withdrawal symptoms, particularly with long-term use (Korpi et al., 2002). Treatment with long-acting benzodiazepine compounds often results in prolonged and unwanted side effects, such as daytime drowsiness associated with hypnotics, and sedation associated with anxiolytics (Kales et al., 1979; Korpi et al., 2002). The use of short-acting benzodiazepine compounds has also been related to unwanted side effects, including anterograde amnesia and psychotic states. It is speculated that a greater knowledge of the basic function and pharmacological relevance of specific $GABA_A$

receptor subtypes will lead to the development of newer generation benzodiazepines (and other classes of allosteric modulators that act at the benzodiazepine binding site- see below) that provide equivalent or even improved efficacy as compared to the classical benzodiazepines, with more targeted actions and fewer side effects (Foster and Kemp, 2006; Rudolph and Mohler, 2006).

The benzodiazepine binding site is the most extensively characterized allosteric modulatory site on the GABA$_A$ receptor. It has been shown that the presence of both an α and a γ subunit is necessary to form the benzodiazepine binding site (Fig. 1-3; Pritchett et al., 1989; Zezula et al., 1996). Differential recognition of classical 1,4-benzodiazepine positive modulators, such as diazepam and flunitrazepam, based on the specific α-subunit isoform present has led to the classification of diazepam-sensitive (DS) and diazepam-insensitive (DI) receptor subtypes (Wieland et al., 1992). DS subtypes include GABA$_A$ receptors where the α_1, α_2, α_3, or α_5 isoform is present, whereas DI subtypes require inclusion of either the α_4 or α_6 isoform. In addition to the benzodiazepines, ligands of numerous classes of compounds bind to this important allosteric modulatory site, including the β-carbolines, imidazobenzodiazepines, and the cyclopyrrolones (Sigel, 2002). Recent isolation of the molluscan acetylcholine binding protein (AChBP) from *Lymnaea stagnalis*, a homopentameric protein that bears homology to the N-terminal domains of the nicotinic acetylcholine receptor subunits, has provided the opportunity for modeling studies of ligand gated ion channel receptors (Smit et al., 2001; Brejc et al., 2001). The structural organization of the benzodiazepine binding site of GABA$_A$ receptors continues to be investigated and recent models based on the structure of the AChBP indicate that the site may be best described as a cleft or pocket within which ligands bind (Cromer et al., 2002). Indeed, molecular modeling using the crystal structure of AChBP has provided clues as to the intersubunit associations of the GABA$_A$ receptor, the secondary structures of the subunits involved, and more specifically, the identity of specific amino acid residues located at the cavity formed by the α-γ interface which may be important to the binding of benzodiazepine site ligands (Grutter and Changeux, 2001; Cromer et al., 2002; Trudell, 2002). Changeux's loop model has also helped clarify the structural organization of the benzodiazepine binding site, having identified that ligand binding domains at subunit interfaces include discrete regions or "loops" of amino acids, with loops A, B, and C contributing from the (+) face of a subunit and loops D, E, and F contributing from the (-) face of the adjacent subunit (Galzi and Changeux, 1994; Changeux and Edelstein, 1998; Fig. 1-3, 1-4). For the benzodiazepine binding site in particular, loops A, B, and C of the α subunit are postulated to interface with loops D, E, and F of the γ subunit (Corringer et al., 2000; Cromer et al., 2002).

Despite these advances, much remains unknown about the structural organization of the benzodiazepine cleft, and how this organization corresponds to functional modulation of $GABA_A$ receptor activity. As mentioned earlier, many different classes of ligands bind at the benzodiazepine binding site. In addition to encompassing a large variety of structural features, these ligands also range the full spectrum of efficacies (Sigel, 2002). It is of value then to understand how binding of these drugs relates to their specific actions. Are there similar binding contacts within the benzodiazepine cleft for all of these compounds regardless of their structure or efficacy? Or are the binding determinants for benzodiazepine site ligands organized in a manner which can be distinguished according to specific characteristics of the allosteric modulator in question? With regard to therapeutic potential, understanding the specificity of drug-receptor interaction at the $GABA_A$ receptor benzodiazepine binding site would greatly enhance the development of pharmacological agents that act at this site with precise and exact effects.

As stated, it is expected that the development of $GABA_A$ receptor subtype-specific therapeutics will minimize the problems associated with the use of classical benzodiazepines (Foster and Kemp, 2006; Rudolph and Mohler, 2006). Evidence gathered from experiments incorporating site-directed mutagenesis hold the greatest potential of providing insight into these types of investigations. This powerful technique allows for specific targeting of a single amino acid residue within the benzodiazepine binding site to undergo point mutation (Davies et al., 2001). Subsequent studies with the mutated $GABA_A$ receptor provide information as to the role and importance of the residue which was mutated to the binding and function of benzodiazepine site ligands. These types of studies have identified residues within the benzodiazepine cleft that are implicated in the activity of allosteric modulators at this site. Histidine 101 (rat α_1) of loop A has been shown to confer sensitivity to classical benzodiazepine positive modulators (Wieland et al., 1992), providing the basis for the DS versus DI classification system. Mutation of this residue to an arginine residue (present in the DI receptor subtypes) in the GABA$_A$ receptor results in a loss of the effects of the benzodiazepine positive modulator diazepam which are normally mediated by this receptor. Genetic studies with point-mutated mice, where individual GABA$_A$ receptor subtypes have been rendered insensitive to diazepam and mice are then observed for a behavioral deficit that might be attributable to loss of diazepam actions at that subtype, have further helped identify which particular actions of diazepam are mediated by a particular receptor subtype (Rudolph and Mohler, 2006). Such investigations have revealed that the sedative actions of diazepam are mediated by α_1-containing GABA$_A$ receptors, while the anxiolytic actions of diazepam are mediated by α_2-containing GABA$_A$ receptors.

Histidine 101 has also been linked to the binding and function of other benzodiazepine site ligands (Fig. 1-5). An array of histidine 101 mutants made previously in this laboratory has been used to show that binding and function of flunitrazepam, Ro15-1788, and Ro15-4513 (a positive modulator, antagonist, and negative modulator respectively) are affected by changes to this residue (Davies et al., 1998; Dunn et al., 1999). Flunitrazepam proves to be particularily sensitive to the nature of the residue replacing histidine 101, showing a dramatic reduction in affinity and efficacy when a charged residue is present. This correlates with experiments with diazepam where GABA$_A$ receptors containing a charged arginine residue in this position were found to be diazepam-insensitive (Wieland et al., 1992; Benson et al., 1998). There is some evidence for specific amino acid residue involvement in the binding of negative modulators and antagonists at the benzodiazepine site as well. Serine 205 (rat α_1) of loop C affects the affinity of the GABA$_A$ receptor for the imidazobenzodiazepines Ro15-1788 and Ro15-4513 (Derry et al., 2004). In particular, a twenty-fold reduction in affinity of the receptor for each of these ligands is observed when serine 205 is mutated to asparagine (Fig. 1-5). Interestingly, no real change in affinity is apparent for flunitrazepam, a benzodiazepine positive modulator. In contrast, two other amino acid residues in the Loop C region, threonine 206 and tyrosine 209, when mutated also resulted in changes in receptor affinity for a number of benzodiazepine-site ligands, including Ro15-1788 and flunitrazepam (Buhr et al., 1997). Taken together, these data have led to the question of whether sub sites exist for certain ligands within the benzodiazepine binding cleft, and whether certain amino acid residues within the loops are linked to the efficacy and/or structure of the ligand being bound. How this relates to the structure of the benzodiazepine cleft itself is still unclear. Improved knowledge of how the benzodiazepine binding cleft is organized will undoubtedly give insight as to the nature of structure-function relationships of this site with the allosteric modulators that bind there.

Classical benzodiazepines continue to be used in the treatment of disorders with underlying excessive excitation, such as anxiety and epilepsy, as well as to induce sleep, muscle relaxation, and anaesthesia (Foster and Kemp, 2006). Nevertheless, the pursuit of newer benzodiazepine site pharmacological agents that will achieve the same therapeutic success, while diminishing the problems associated with side effects due to non-selective actions, remains significant. Subtype-selective benzodiazepine site therapeutics with the potential for fewer side effects that are currently being researched include non-sedating anxiolytics (α_2- or α_3-selective), and hypnotics (α_1-selective) (Dämgen and Lüddens, 1999; Atack et al., 2005; Rudolph and Mohler, 2006). Interestingly, the development of cognitive enhancers associated with selective negative modulation at α_5-containing $GABA_A$ receptors is also being investigated (Sternfeld et al., 2004; Chambers et al., 2004). While recent advances in the knowledge of the structure of the $GABA_A$ receptor benzodiazepine binding site has helped clarify the basic organization of the cleft and identified residues which are important to ligand binding and function at this site, much remains to be elucidated as to the exact mechanism by which benzodiazepine site allosteric modulators produce their effects. The heterogeneity of $GABA_A$ receptor subtypes, combined with the diversity of ligands with respect to structure and efficacy, will certainly continue to provide valuable opportunities for the advancement of $GABA_A$ receptor benzodiazepine site therapeutics.

5. GABA-site agonist pharmacology

While the benzodiazepine binding site of the $GABA_A$ receptor is perhaps the most characterized site of the receptor, interest continues to increase with regard to the potential for therapeutics that act via the endogenous agonist site itself. Gaboxadol

(THIP) is currently in late-stage investigations as a treatment for insomnia (Wafford and Ebert, 2006). Unlike the benzodiazepines, however, which largely target $GABA_A$ receptors located at the postsynaptic membrane, THIP is more selective for extrasynaptic $GABA_A$ receptors. It is this functional selectivity which has renewed interest in GABA site agonists as possible therapeutic agents.

GABA binding sites are predicted to be present at each β(+)-α(-) subunit interface of the $GABA_A$ receptor (Smith and Olsen, 1995). These low affinity sites are presumed to be involved in channel activation, in agreement with the micromolar concentrations of GABA required for receptor activation in functional studies. Molecular modeling using the crystal structure of AChBP has also helped elucidate the structure of the GABA agonist binding site (Cromer et al., 2002). Similar to the benzodiazepine binding site, residues from loops A, B, and C of the β subunit contribute along with loops D, E, and F of the α subunit. There is also evidence suggesting the existence of a separate high affinity GABA agonist binding site (Newell et al., 2000). Speculated to be present at other subunit-subunit interfaces, such as the α(+)-β(-) interface, it has been suggested that binding of the agonist at this high affinity site may play a role in stabilizing the desensitization state of the receptor (Newell and Dunn, 2002).

As mentioned, extrasynaptic $GABA_A$ receptors likely contain mainly α_4, α_5, α_6, and δ subunits (Mody, 2001; Whiting, 2003). In contrast to the subtype selectivity based on binding affinity seen with the benzodiazepine site ligands, the affinity of $GABA_A$ receptor agonists is not chiefly dependent on subunit composition (Wafford and Ebert, 2006). The amino acids across the isoforms of the α and β subunits are highly conserved, and as the agonist binding site is located at their interface, lack of selectivity based on this criterion is not surprising. Rather, functional selectivity based on the extrasynaptic location of $GABA_A$ receptors containing specific subunits seems a more likely target for pharmacological intervention. For instance, it is suggested that the presence of the δ subunit, which is thought to replace the γ subunit in the pentamer, is associated with localization to extrasynaptic areas (Nusser et al., 1998). Interestingly, it has been shown that THIP acts with higher potency and efficacy at δ-containing $GABA_A$ receptors, as compared to other receptor subtypes (Brown et al,. 2002; Wafford and Ebert, 2006). The development of other GABA site agonists or partial agonists with similar properties could provide a unique mechanism for targeting overall tonic inhibition of the brain (see Chapter 6). Although this regulation of neuronal excitability remains poorly understood, it is proposed that extrasynaptic receptors respond to spillover of synaptically-released GABA, non-vesicular released GABA, or ambient levels (Cavelier et al,. 2005). Pharmacological agents selective for extrasynaptic $GABA_A$ receptors may therefore reveal novel therapeutic targets for the treatment of various disorders affected by underlying imbalances in excitation and inhibition.

6. Aims of present studies

The overall aim of the present studies is to investigate ligand interaction at specific $GABA_A$ receptor subtypes. Radioligand binding techniques as well as two-electrode voltage clamp electrophysiology provides the ability to perform both affinity studies and functional assays to characterize ligand interaction (see Chapter 2). The use of site-directed mutagenesis allows for the comparison of recombinant wild type versus mutant $GABA_A$ receptors. The present studies focus on either the benzodiazepine binding site or the GABA agonist binding sites of different $GABA_A$ receptor subtypes.

Numerous studies have demonstrated that the flavonoids, a class of compounds found in all vascular plants, have affinity for the benzodiazepine binding site of the $GABA_A$ receptor (Nielsen et al., 1988; Medina et al., 1989; Medina et al., 1997; Viola et al., 1999; Dekermendjian et al., 1999; Marder et al., 2001; Kahnberg et al., 2002; Marder and Paladini, 2002; Butterweck et al., 2002). Chapter 3 focuses on the interaction of amentoflavone, a biflavonoid constituent of St. John's Wort, with recombinant wild type and mutant $GABA_A$ receptors. The aim of the study was to characterize the mechanism of interaction of amentoflavone with the benzodiazepine binding site and to determine whether the functional effects of this biflavonoid at the $GABA_A$ receptor are indeed mediated by this site. Flavonoid interaction at the $GABA_A$ receptor is also focused on in Chapter 4. The binding profile and functional effects of the high affinity synthetic flavonoid derivative 5′-bromo-2′-hydroxy-6-methylflavone at different $GABA_A$ receptor subtypes is investigated. The possible structural requirements for high affinity flavonoid binding at the benzodiazepine binding site are also discussed.

As mentioned, the array of histidine 101 mutants made previously in this laboratory has been used to demonstrate that the binding and function of flunitrazepam, Ro15-1788, and Ro15-4513 (a positive modulator, antagonist, and negative modulator respectively) are affected by mutation of this residue (Davies et al., 1998; Dunn et al., 1999). The effects of various point mutations of this residue on the affinity and efficacy of these benzodiazepine site ligands were compared. In a similar manner, Chapter 5 focuses on the efficacy changes observed for a β-carboline benzodiazepine site ligand, ethyl-β-carboline-3-carboxylate (β-CCE), at the array of histidine 101 mutant receptors as compared to the wild type $GABA_A$ receptor. These changes in efficacy are also compared to changes in affinity of β-CCE seen at the same mutant receptors (Derry, unpublished observations), and possible implications with regard to ligand binding at the benzodiazepine site are discussed.

With increasing interest in the potential for the development of therapeutic agents that target extrasynaptic $GABA_A$ receptors to affect overall tonic inhibition, further research into the differential effects of GABA-site agonists at different receptor subtypes to determine the significance of specific subunits is important. It has been shown that the partial agonists THIP and P4S produce dramatically different responses at the diazepam-insensitive, α_4- versus α_6-containing $GABA_A$ receptors (Wafford et al., 1996). The changes in agonist potency and efficacy were observed despite the high sequence homology between these two subunits, both of which are thought to be present in extrasynaptic $GABA_A$ receptors. In Chapter 6, the agonist responses of GABA, THIP, and P4S are studied at α_4- and α_6-containing $GABA_A$ receptor subtypes. Chimeric α_6/α_4 subunits constructed previously in this laboratory (Derry et al., 2004), are also compared to determine whether a specific domain can be identified which confers differential sensitivity to the subunits.

In conclusion, the focus of this research is to characterize the structure-function relationship of the benzodiazepine binding site, and its interaction with allosteric modulators of the $GABA_A$ receptor. Emphasis is placed on identifying specific binding determinants involved in the binding and function of various classes of benzodiazepine site ligands, such as the flavonoids. Additionally, the possible differential sensitivity of GABA-site agonists at diazepam-insensitive $GABA_A$ receptor subtypes is also investigated to clarify the potential importance of the α-subunit isoform present in extrasynaptic receptors. Ultimately, this research aims to better characterize ligand interaction at the benzodiazepine and GABA binding sites, and the mechanism by which these ligands affect the overall activity and function of the $GABA_A$ receptor. The $GABA_A$ receptor remains a key target for pharmacological intervention in the search for improved therapies in the treatment of various psychiatric and neurological disorders. It is hoped that enhanced knowledge of ligand interaction at this complex receptor, and the increased potential for selective targeting provided by the vast heterogeneity associated with its many subtypes, will further advance the discovery of such drugs.

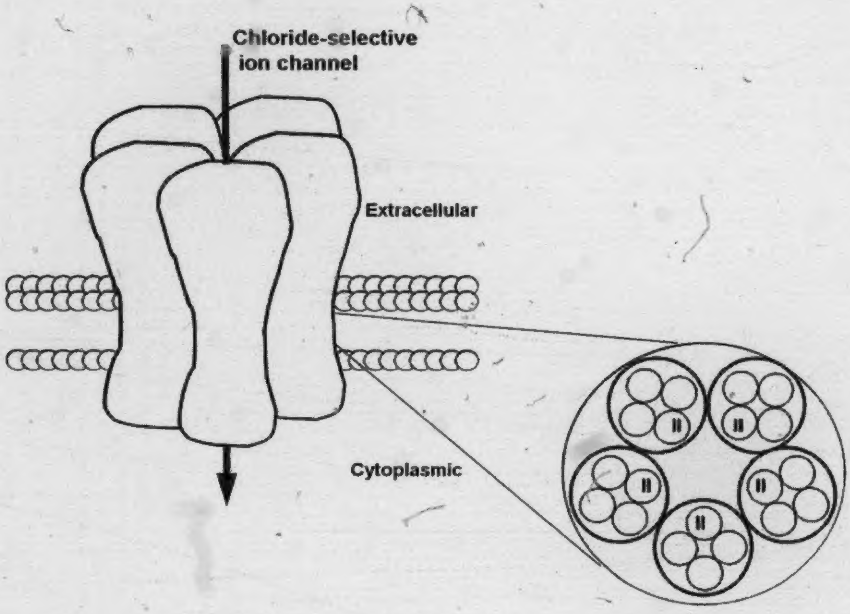

Figure 1-1

A representation of the $GABA_A$ receptor within the plasma membrane. The central chloride-selective ion channel is indicated. A schematic planar view of the heteromeric pentamer, with the five subunits surrounding the ion channel pore, is magnified. Note that transmembrane domain II of each of the five subunits lines the channel pore (see Figure 1-2).

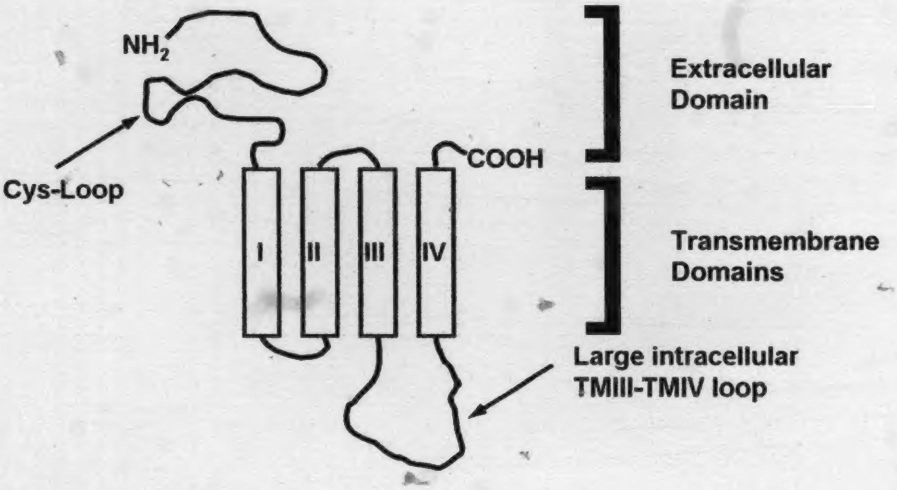

Figure 1-2

The topology of a single GABA$_A$ receptor subunit is depicted. Basic structural homology shared by the subunits includes a large extracellular N-terminal domain, four transmembrane domains, and a small extracellular C-terminal domain. The "cys-loop" formed by conserved cysteine residues linked by a disulfide bond that is characteristic of all subunits of the ligand-gated ion channel superfamily is shown. Note the large intracellular loop between transmembrane domains III and IV.

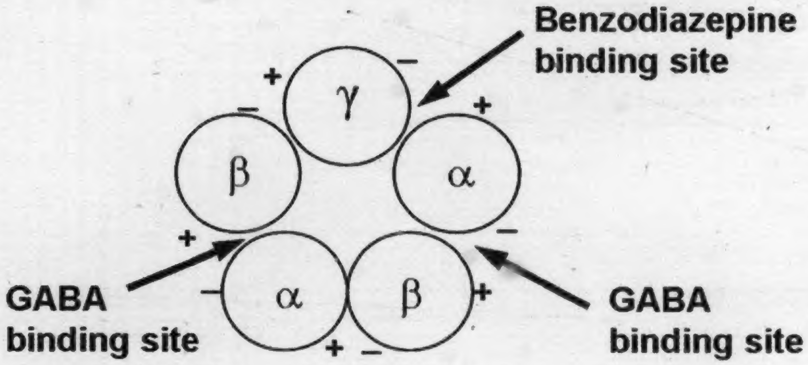

Figure 1-3

A depiction of the $GABA_A$ receptor heteropentamer. The subunit composition containing α, β, and γ subunits, thought to comprise the majority of $GABA_A$ receptor subtypes, is shown. The benzodiazepine binding site is located at the α(+)/γ(-) subunit interface. The agonist (GABA) binding sites are located at each of the β(+)/α(-) subunit interfaces.

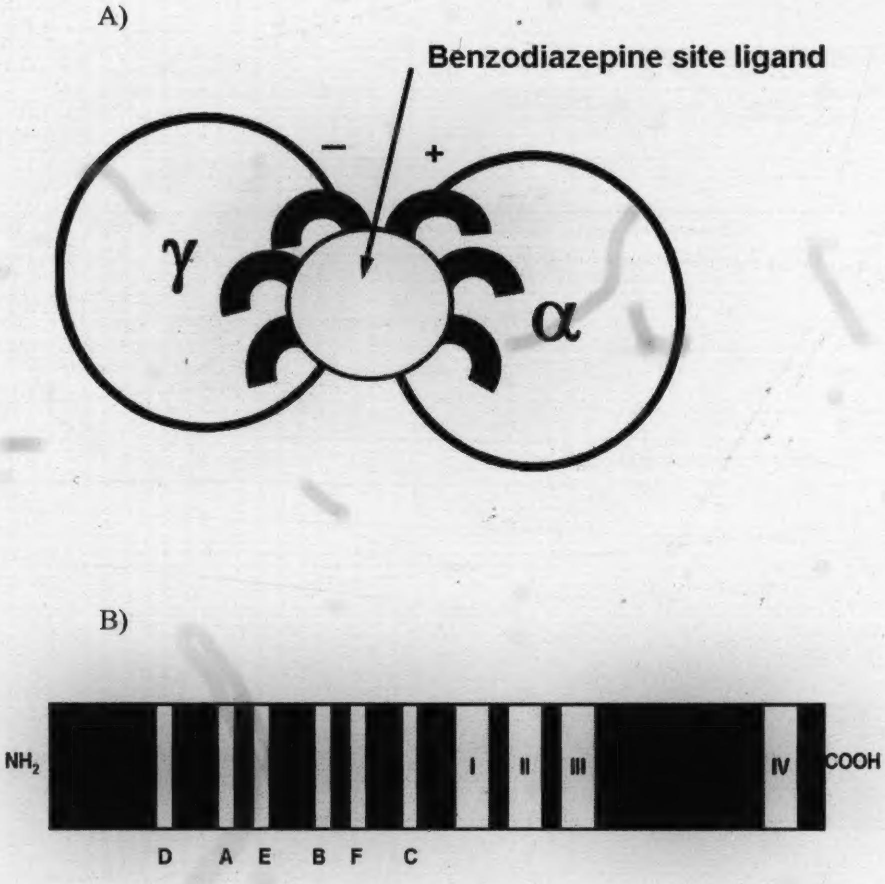

Figure 1-4

A) A cartoon representation of the benzodiazepine binding site located at the α(+)/γ(-) subunit interface. The "loops" or discrete regions of the N-terminal domains, postulated to contribute to ligand binding, are shown interacting with a benzodiazepine site ligand.

B) A linear model of a single GABA$_A$ receptor subunit. Segments encoding loops A-C, found on the (+) face of each subunit, and loops D-F, found on the (-) face of each subunit, are identified along the N-terminal section of the amino acid sequence, followed by identification of segments encoding the four transmembrane domains.

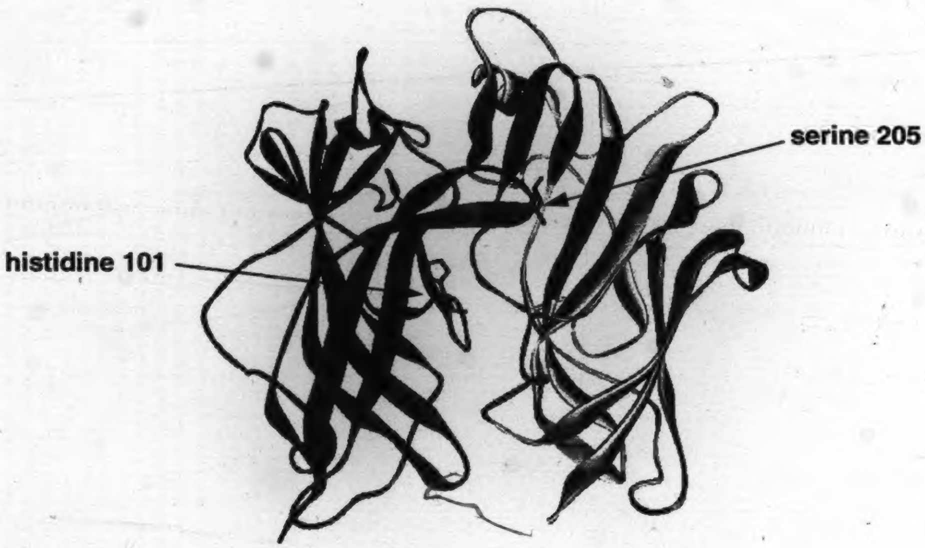

Figure 1-5

A model of the benzodiazepine binding site located at the $\alpha(+)/\gamma(-)$ subunit interface based on comparative modeling with the atomic structure of the AChBP (Ernst et al., 2003). Loops A (*red*), B (*aqua*), and C (*green*) of the α subunit and loops D (*blue*), E (*magenta*), and F (*orange*) of the γ subunit are indicated. The relative positions of the histdine 101 and serine 205 amino acid residues, located within loop A and loop C of the α subunit, respectively, are also depicted (see Benzodiazepine Pharmacology discussion). Note that a more recent model of the structure of the $GABA_A$ receptor which incorporates the transmembrane domains is available (Ernst et al., 2005).

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CHAPTER 2

Materials and Methods

1. Transfection of cells and preparation of membrane homogenate

Transient transfection of tsA 201 cells was performed using a calcium phosphate transient transfection protocol (Chen and Okayama, 1988; Newell et al., 2000). Derived from the human embryonic kidney 293 (HEK 293) cell line, the tsA 201 cell line expresses the simian virus 40 (SV40) T-antigen at high levels, thereby promoting replication of plasmids containing the SV40 origin of replication (Newell et al., 2000). High levels of $GABA_A$ receptor expression can therefore be achieved using this cell line. Cells were maintained in 15 cm diameter plates in 20 ml of low-glucose Dulbecco's modified Eagle's medium [Hyclone, Logan, Utah] containing 10% fetal calf or bovine growth serum and stored at 37°C in a 7% CO_2 humidified incubator. Cells were passaged twice a week.

cDNAs (rat) encoding the subunits of interest were subcloned into the pcDNA3.1 (+/-) expression vector [InVitrogen, San Diego, CA] and transformed into competent *Escherichia coli* cells for long-term maintenance and propogation of the plasmid DNAs. Subsequent plasmid purification to isolate the plasmid DNA from the bacterial strain was carried out using DNA maxipreps (Qiagen, Mississauga, ON). Briefly, individual colonies were isolated from bacterial culture streaked on ampicillin-selective plates, incubated for ~6 h at 37°C in Luria Bertani (LB) medium containing ampicillin with vigorous shaking to obtain a starter culture, and subsequently diluted 1/1000 into ampicillin selective LB medium and grown overnight for ~16 h at 37°C with vigorous shaking. Overnight cell suspension was transferred to centrifuge tubes and centrifuged at 6000*g* for 15 min at 4°C. The bacterial pellet was resuspended in 10 ml of resuspension buffer and vortexed to completely dissolve cell clumps. Addition of 10 ml of cell lysis buffer to the suspension was followed by vigorous inverting of the sealed tube 4-6 times and incubation on ice for 5 min. Subsequent addition of 10 ml of neutralization buffer was also followed by vigorous inverting of the sealed tube 4-6 times and 20 min incubation on ice to precipitate genomic DNA, proteins and cell debris. The sample was then centrifuged at >20,000*g* for 30 min at 4°C and the resulting plasmid-containing supernatant applied to an equilibrated resin column via gravity flow. A wash buffer was added to the column to remove any remaining contaminants and the plasmid DNA was then eluted with an elution buffer into another centrifuge tube. DNA was precipitated by addition of isopropanol followed by centrifugation at >15,000*g* for 30 min at 4°C. The supernatant was decanted and the DNA pellet washed by addition of 70% ethanol followed again by centrifugation at >15,000*g* for 15 min at 4°C. The supernatant was decanted and the DNA pellet allowed to dry before finally being redissolved in 250 µl of sterile water, transferred to a 1.5 ml microfuge tube, and stored at -20°C. The above described protocol generally yielded 300-800 µg of purified plasmid DNA, and final plasmid cDNA concentration was determined using absorbance, where a 1:1000-2000 dilution of DNA was read at a wavelength of 260 nm (with one unit A_{260} equivalent to 50 µg/ml DNA).

cDNAs encoding the subunits of interest were added in equal amounts (10 µg of each subunit for each plate to be transfected) to an appropriate volume of 250 mM $CaCl_2$ (0.5 ml for each plate to be transfected), followed by addition of an equal volume of N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer [50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.0. Note that smaller-scale pH optimization trials evaluating a pH range from 6.98 to 7.02 were carried out prior to large-scale transient transfections being performed in order to determine the optimum pH for the BES buffer. Typically, it was found that a pH of 7.00 or 7.02 yielded the highest expression levels]. Contents were mixed and allowed to stand for 5 min at room temperature before 1 ml aliquots were added dropwise to plates of cells that had reached optimum density for transfection. Cells were then incubated at 37°C in a 3% CO_2 humidified incubator for 48 h, and media was replaced with fresh media 24 h following transfection. Cells were harvested, following removal of media, into a Tris-HCl solution [50 mM Tris, 250 mM KCl, 0.02% NaN_3, pH 7.4, supplemented with 1 mM benzamidine, 0.1 mg/ml bacitracin, 0.01 mg/ml chicken egg white trypsin inhibitor, and 0.5 mM phenylmethylsulfonyl fluoride] and sheared using an UltraTurrax T25 homogenizer [IKA Labortechnik, Staufen, Germany] (13,500 rpm for two 10 s pulses). Homogenized cells were pelleted via centrifugation (39,100*g* for 30 min). The supernatant was decanted and the remaining pellet resuspended in Tris-HCl solution and transferred to 1.5 ml microfuge tubes. The tubes were briefly vortexed and microfuged at ~15,000*g* for 1 min at room temperature to pellet cellular debris. The supernatant, containing membrane fragments, was then isolated and stored at -80°C.

Rat brain membrane homogenate was prepared as follows. Briefly, the brains (three brains per centrifuge tube) were homogenized in Tris-HCl harvesting solution using an UltraTurrax T25 homogenizer [IKA Labortechnik, Staufen, Germany] at low speed, then high speed for 10 s. The homogenate was topped up with Tris-HCl solution and then centrifuged for 10 min (SS34 rotor; 2,500 rpm). The supernatant was transferred to a fresh centrifuge tube. The centrifuge tube containing the original pellet was topped up with 10 ml of Tris-HCl solution, homogenized, and centrifuged as before. The supernatant was pooled with the initial supernatant sample and the pellet discarded. The centrifuge tube containing the supernatant was topped up with Tris-HCl solution and centrifuged for 20 min (Beckman type 45Ti rotor; 40,000 rpm) and the supernatant discarded. The resulting pellet was resuspended in 10 ml of Tris-HCl solution, homogenized, the centrifuge tube topped up with Tris-HCl solution and centrifuged for 20 min (Beckman type 45Ti rotor; 40,000 rpm). This wash step was repeated two more times. The final pellet was resuspended in 20 ml of Tris-HCl solution. Protein content was assessed using the Biorad Protein Assay (Biorad, Hercules, CA) and membranes diluted to a final concentration of 0.3 mg/ml. Membranes were stored at -80°C for use in experiments.

2. Radioligand binding assays

Radioligand competition binding experiments were performed as described previously (Davies et al., 1998; Newell et al., 2000; Derry et al., 2004). It should be noted that prior to performing large-scale experiments, binding checks comparing background, total, and nonspecific radioactivity counts were performed on membrane homogenate to confirm that receptor expression levels were adequate. Generally, background counts were found to be <1% of total counts and nonspecific binding counts were found to be ~3-10% of total counts. Average total binding counts achieved ranged from 1000-4000 counts per minute. For large-scale radioligand competition binding experiments, aliquots of cell homogenates were incubated with [^{3}H]Ro15-4513 (specific activity 20-40 Ci/mmol) or [^{3}H]Ro15-1788 (specific activity 70-90 Ci/mmol) in a Tris-HCl buffer [50 mM Tris, 250 mM KCl, 0.02% NaN_3, pH 7.4] in a final volume of 500 µl for 1 h at 4°C. GF/B filters [Whatman, Maidstone, UK] to be used were also soaked in

Tris-HCl buffer at 4°C for 1 h. Nonspecific binding was determined in the presence of excess unlabelled ligand (100-150 μM). The mixture was filtered using a cell harvester [Brandel, Gaithersburg, MD] and washed twice with 5 ml of ice-cold Tris-HCl buffer. Filters were then dried under a heat lamp and placed into scintillation minivials. CytoScint scintillation fluid [ICN, Costa Mesa, CA, 5 ml] was added to each vial and radioactive decay was measured by liquid scintillation counting.

3. Transcript preparation and injection of oocytes

The histidine 101 array of mutants was created via site-directed mutagenesis as described previously (Davies et al., 1998). Briefly, the α_1 subunit was subcloned into the pAlter-1 vector using the Altered Sites kit [Promega, Madison, WI]. The presence of a restriction site, introduced by the mutagenic oligonucleotide as a silent mutation, was used to identify potential mutants. Correct substitutions were verified by DNA sequencing, and mutant α_1 cDNAs were then subsequently subcloned into the pcDNA 3.1 expression vector [InVitrogen, San Diego, CA].

Randomly derived chimeras from the α_4 and α_6 subunits were created following the protocol of Moore and Blakely (1994) as described previously (Derry et al., 2004). To summarize, the complete α_6 cDNA was inserted (as a *Hind*III fragment) into pcDNA 3.1 (+) expression vector [InVitrogen, San Diego, CA], upstream of a partial α_4 cDNA sequence (an *Eco*RI-*Xba*I fragment containing an N-terminally truncated α_4 sequence). The polylinker between the inserts contained a *Bam*HI restriction site. To limit the amount of α_6 insert that was homologous to the α_4 cDNA, restriction digestion was used to excise the intervening polylinker as well as a portion of the α_6 insert. Restriction digestion of the dual plasmid with BamHI and HpaI or with EcoRI and BstXI limited the region homologous with the α_4 insert to a sequence in the α_6 cDNA that represented the extracellular N-terminus of the mature α_6 subunit, thereby targeting the random crossover events to occur in regions of the cDNA that represent extracellular ligand binding domains of the α subunits (Boileau et al., 1998). Random crossover events occurred at regions of homology in the α-subunit sequences following transformation of the linearized plasmid DNA into Library Efficiency® competent DH5α *Escherichia coli* cells [Life Technologies, Gaithersburg, MD], to produce in-frame hybrid cDNA. Plasmid

DNA was extracted from isolated individual colonies and for those showing a chimeric crossover, the switch point was assessed via restriction digest analysis and confirmed by DNA sequencing. Chimeras were named based on the point of crossover with the number representing the last residue of N-terminal α_6-subunit contribution (rat subunit numbering).

cRNA transcripts were prepared by standard protocol, as described previously (Hope et al., 1993; Kapur et al., 2006 in press). cDNAs in the pcDNA 3.1 vector, encoding chimeras, α, β, or γ subunits, were linearized by setting up a restriction digest using 10 μg of DNA in a final reaction volume of approximately 10 μl with an appropriate restriction enzyme. The reaction was mixed and incubated in a 37°C water bath for 1 h (temperature and incubation time varied with type of enzyme used). Following DNA template linearization, an *in vitro* RNA transcription reaction was set up, with special care taken to avoid RNase contamination by using gloves, sterile glassware, and water devoid of RNase activity [treated with 0.1% diethyl pyrocarbonate (DEPC) and autoclaved]. The reagents required for transcript preparation are listed in Table 2-2. The reaction was set up on ice by adding first DEPC-treated water to a 1.5 ml microfuge tube, and subsequently all other components listed in Table 2-2, except the RNA polymerase. Next, 50 U of RNA polymerase was added to the reaction, and the sample gently mixed by flicking, microfuged for a few seconds, and incubated in a 37°C water bath for 1 h. An additional 50 U of RNA polymerase was added and the reaction incubated for another 1 h at 37°C. To degrade the template DNA following the transcription reaction, 5 U of RNase-free DNase was added to the reaction mix and the sample was incubated at 37°C for 15 min. The RNA transcript was then extracted from solution by first adding DEPC-treated water to bring the final volume to 400 μl, and subsequently adding 400 μl of 25:24:1 (v/v) phenol/chloroform/isoamyl alcohol to the reaction mix (in a fumehood). The tube was briefly vortexed and microfuged at ~15,000*g* for 2 min at room temperature to separate the aqueous from the organic phase. The upper (aqueous) layer was then carefully transferred into another 1.5 ml microfuge tube. RNA transcript was precipitated by addition of 1:10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold 100% ethanol, with the sample mixed and placed at -80°C for at least 1 h. The tube was then microfuged at ~15,000*g* for 15 min at 4°C to collect the RNA pellet. Supernatant was carefully discarded so as not to dislodge the pellet and 500 μl of 70% ethanol (in DEPC-treated water) added to the tube. The tube was microfuged again for 5 min at ~15,000*g* at room temperature and the pellet subsequently allowed to air dry for about 5 min before being resuspended in 10 μl of DEPC-treated water. The concentration of cRNA transcript was determined by absorbance, where a 1:1000-2000 dilution of transcript was read at a wavelength of 260 nm (with one unit A_{260} equivalent to 40 μg/ml RNA), and absorbance ratio was used to assess for purity ($A_{260}/A_{280}>1.6$) and phenol contamination ($A_{260}/A_{230}>2.0$). cRNA transcript was then diluted to a final concentration of 1 μg/μl in DEPC-treated water and stored at -80°C.

cRNA transcripts combinations in a 1:1:1 ratio (1.0 μl of cRNA encoding each required subunit) were added to a 1.5 ml microfuge tube and kept on ice until ready for injection. A glass pipette tip (10 μl Drummond Microdispenser 100 replacement tubes, Broomall, PA) was pulled using a one-stage magnetic puller with a heating element, and the end of the injecting tip subsequently scored using flame-heated forceps. The glass pipette was then filled with mineral oil that had been dyed with 4-amino-3-nitrotoluene (MP Biomedicals, Irvine, CA) to check that the tip was patent. The glass pipette was attached to a microdispenser (10 μl digital, Drummond, Broomall, PA) which was then mounted onto a micromanipulator. Under a microscope, the transcript solution droplet placed on Parafilm was carefully drawn into the microinjector tip (with care take to avoid drawing air bubbles into the glass pipette tip as these interfere with RNA injection). cRNA transcript was then injected into stage V and VI *Xenopus laevis* oocytes that had been defolliculated by treatment with collagenase A [2 mg/ml, Beohringer-Mannheim, Indianapolis, IN] for 3 h at room temperature. Care and handling of *Xenopus* frogs was in accordance with approved guidelines by the Canadian Council on Animal Care, and the Biosciences Animal Policy and Welfare Committee of the University of Alberta (Smith et al., 2004). Oocytes were transfered to a petri dish (with a small piece of mesh attached to the bottom to hold the oocytes in place) filled with standard ND96 solution [96 mM NaCl, 5 mM HEPES, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4] supplemented with 0.1 mg/ml gentamycin. The manipulator was then used to gently puncture the membrane and cRNA transcript was injected [50 nl of 1 mg/ml cRNA] into the oocyte vegetal pole. It was advantageous to wait a few seconds before slowly withdrawing the tip to minimize leakage of oocyte contents and cRNA transcript through the puncture site. The injected oocytes were individually maintained at 14°C for up to 14 days in 96-well plates containing 200 μl of standard ND96 solution supplemented with 0.1 mg/ml gentamycin.

4. Electrophysiological recordings

Following a 48 h incubation period, injected oocytes were used for electrophysiology experiments for up to 14 days. The experimental method was similar to that described previously (Pistis et al., 1997; Dunn et al., 1999; Kapur et al., 2006 in press). Briefly, a GeneClamp 500 Amplifier [Axon Instruments, Inc., Foster City, CA] was used to hold the membrane potential of an oocyte in the bath chamber at -60 mV, via the two-electrode voltage-clamp mode. Current is passed via an electrode to clamp the membrane potential, and a second voltage-sensing electrode is used to measure the actual membrane potential, with the amplifier allowing for rapid readjustment and maintenance of the command voltage clamp. The chamber (0.5 ml) in which the oocytes were held was supplied with a constant flow of ND96 perfusion buffer [96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4] using a gravity-flow drip system. The voltage-sensing and current-passing electrodes were filled with 3 M KCl such that they had a resistance of 0.5-3.0 MΩ as measured in ND96 perfusion buffer. The electrical signal from the amplifier was transduced to a digital signal that was fed into a computer by a Digidata 1322A [Axon Instruments, Inc., Foster City, CA] data acquisition system. Current response to drug application over a time interval was obtained as a current trace that could be analyzed using the Axoscope 9.0 [Axon Instruments, Inc., Union City, CA] acquisition program.

For all $GABA_A$ receptor subtypes studied, the effects of modulators [amentoflavone; 5′-bromo-2′-hydroxy-6-methylflavone; ethyl β-carboline-3-carboxylate (β-CCE)] were investigated using a GABA concentration that elicited a current approximately 50% (EC_{50}) of the maximal GABA response (which had been established by determining the GABA concentration-effect relationship at each particular subtype; see Data analysis below), with the exception of the experiments testing modulator block of diazepam positive modulation of GABA-evoked current, where a 3 μM concentration of GABA was used. Modulator stock concentrations were made by dissolving the compounds in dimethyl sulfoxide, and stocks were stored at room temperature under light-sensitive conditions. In all experiments, the concentration of dimethyl sulfoxide was kept constant at a concentration ($\leq$0.01%) that produced no overt vehicle effects. Control currents using an EC_{50} concentration of GABA were first recorded until current amplitude was consistent to within ±5% over three successive challenges, with a 12 min wash with perfusion buffer allowed between each application to permit recovery. A 3 min perfusion of modulator was followed by coapplication of the compound with an EC_{50} concentration of GABA. This protocol was also followed for application of diazepam and its coapplication with a 3 μM concentration of GABA. Evoked current was recorded, and a 12 min wash with perfusion buffer was again allowed before the next application of modulator. Control responses to the EC_{50} concentration of GABA were recorded periodically throughout each experiment to confirm that current response remained stable, and that the effects of modulator application were reversible.

The effects of agonists [GABA; 4,5,6,7-Tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol hydrochloride (THIP); Piperidine-4-sulphonic acid (P4S)] were investigated by first stabilizing current amplitude elicited by a saturating concentration of GABA (3 mM), measured as a maximal response in all the recombinant $GABA_A$ receptor subtypes studied, such that it remained consistent to within ±5% over three successive challenges. During stability measurements, and upon recording currents to determine the concentration-effect relationship, a 12 min wash with perfusion buffer was allowed between applications of agonist concentrations to permit recovery. Control responses to the saturating concentration of GABA were recorded periodically throughout each experiment to confirm that current response remained stable. Agonist stock concentrations were made by dissolving the compounds in ND96 perfusion buffer, and were prepared fresh upon commencement of each experiment.

5. Data analysis

Data from competition binding assays were fit to curves using least-squares non-linear regression analysis of GraphPad Prism 3.0 [GraphPad, San Diego, CA]. K_d values for the radioligand at wild type and mutant receptor subtypes examined are summarized in Table 2-1. Log IC_{50} values were determined as mean ± S.E.M. of at least three independent experiments. K_i values were determined using the equation of Cheng and Prusoff (1973). One-way analysis of variance between groups (ANOVA) was used for data analysis and levels of significance were determined using Dunnett's post test.

Data analysis was performed by means of nonlinear regression techniques using GraphPad Prism 3.0 [GraphPad, San Diego, CA]. The GABA concentration-effect relationship of recorded current was fit by the equation:

$$I = \frac{I_{\max} * [L]^n}{\mathrm{EC}_{50}{}^n + [L]^n}$$

where I is the measured amplitude of evoked current, [L] is the GABA concentration, EC_{50} is the GABA concentration that produces half the maximal response ($I_{\max}$), and n is the Hill coefficient. Concentration-effect curves were determined by normalizing the evoked current to the $I_{\max}$ (100%). Concentration-effect curves for THIP and P4S were determined using the same equation and by normalizing the evoked current to that produced by a saturating concentration of GABA (3 mM), representing I_{max} (100%).

The effects of modulators were fit by one-site binding analysis using the equation:

$$I = I_o + \frac{(I_{\mathrm{GABA}} - I_o)}{(1 + 10^{x-c})}$$

where I is the measured amplitude of evoked current, I_{GABA} is the current in response to EC_{50} GABA concentration in absence of modulator, I_o is the current in the presence of both EC_{50} GABA concentration and a saturating concentration of modulator, X is the logarithm of modulator concentration, and C is the logarithm of the IC_{50} concentration. Data are presented as log IC_{50} ± S.E.M. and mean with 95% confidence intervals of observations made from at least three oocytes.

6. Drugs and cDNA

[^{3}H]Ro15-4513 and [^{3}H]Ro15-1788 were obtained from Perkin-Elmer (Boston, MA). GABA, THIP, and P4S were obtained from Sigma Chemical Co. (St. Louis, MO). Amentoflavone was obtained from Indofine Chemical Co. (Hillsborough, NJ). 5′-Bromo-2′-hydroxy-6-methylflavone was a gift from Dr. T. Liljefors of the Royal Danish School of Pharmacy. Diazepam was a gift from Dr. G. Baker of the Department of Psychiatry at the University of Alberta. β-CCE was a gift from Dr. B. Jones of GlaxoSmithKline (Harlow, UK).

cDNA encoding α, β, and γ $GABA_A$ receptor subunits were a gift from Dr. P. H. Seeburg of the University of Heidelberg (Germany). cDNA encoding the $GABA_A$ receptor δ subunit was a gift from Dr. R. L. MacDonald of the Vanderbilt University Medical Center (Nashville, TN). cDNA encoding $5HT_3$ receptor subunits were a gift from Dr. E. F. Kirkness of the Institute for Genomic Research (Rockville, MD).

GABA$_A$ Receptor Subtype	Affinity
α subunit	**K$_d$ (nM)**
$\alpha_1\beta_2\gamma_2$	5.4 ± 0.5[a]
$\alpha_2\beta_2\gamma_2$	20.5 ± 2[b]
$\alpha_4\beta_2\gamma_2$	6.4 ± 0.4[c]
$\alpha_5\beta_2\gamma_2$	0.2 ± 0.05[a]
$\alpha_6\beta_2\gamma_2$	5.7 ± 0.7[c]
rat*	0.9 ± 0.1[a]
α_1 subunit histidine 101 mutation	
tyrosine	7.0 ± 0.7[d]
lysine	1.5 ± 0.1[d]
glutamate	2.1 ± 0.1[d]
glutamine	0.49 ± 0.07[d]
alanine	8.3 ± 0.5[a]

Table 2-1

K$_d$ values for the radioligand [^{3}H]Ro15-4513 at wild type and mutant GABA$_A$ receptor subtypes. Values are listed for wild type receptors containing different α subunits, and mutant receptors containing an amino acid substitution at position 101 of the α_1 subunit. Data are from at least three experiments performed in duplicate.

*[^{3}H]Ro15-1788 was used as the radioligand for competition assays performed using rat brain membrane preparations. [a]Derry, unpublished data. [b]Paulsen, unpublished data. [c]Derry et al., 2004. [d]Davies et al., 1998.

***In vitro* transcription reagents**
5x transcription buffer (1:5 final reaction volume)
60 U RNase Inhibitor
10mM DTT (dithiothreitol)
0.5mM 7-methyldiguanosine triphosphate [mG(5′)PPP(5′)G] RNA capping analog
0.5mM NTP mix (UTP, ATP, GTP, CTP)
5.0µg linearized DNA template
100 U T7-RNA polymerase
0.1% DEPC-treated water to bring reaction to 50µl final volume

Table 2-2

Components required for *in vitro* RNA transcription preparation (adapted from Kapur et al., 2006 in press). The reagents required are available through numerous companies including InVitrogen (Burlington, ON), New England Biolabs (Pickering, ON), and Promega (Madison, WI).

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CHAPTER 3*

Determinants of Amentoflavone Interaction at the GABA$_A$ Receptor

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Data from competition binding experiments performed with amentoflavone tested at rat brain membrane, wild type $\alpha_1\beta_2\gamma_2$, and mutant GABA$_A$ receptor subtypes was contributed by I. Paulsen. Functional data for amentoflavone tested at 5HT$_3$ receptor subtypes were from experiments performed in collaboration with I. Paulsen.

1. Introduction

St. John's Wort (*Hypericum perforatum*) is a herbal remedy which is increasingly being used in the treatment of mild to moderate depression (Cott and Fugh-Berman, 1998; Nathan, 1999; Greeson et al., 2001). Historically, this plant has been used for centuries to treat ailments as diverse as burns, inflammation of the skin, gastroenteritis, ulcers, diarrhea, and hysteria. Usually classified as a dietary supplement, herb or natural product, this nonprescription drug is now widely indicated for internal use as a treatment for mild to moderate depression, and external use in healing wounds, treating first-degree burns, and relieving myalgia (Blumenthal et al., 2003). St. John's Wort continues to gain popularity in North American markets as a natural remedy; however its status as an effective antidepressant remains particularly strong in Germany, where the herbal extracts are frequently prescribed by healthcare providers (Cott and Fugh-Berman, 1998; Nathan, 1999; Blumenthal, 2003). Nevertheless, despite these widespread indications and the growing use of this plant to treat depression in recent years, there remains little resolution as to the identity of the active constituents or the mechanism(s) behind this antidepressant effect.

St. John's Wort contains numerous biochemical classes of compounds including the naphthodianthrones (hypericin, pseudohypericin), the phloroglucinols (hyperforin), and the flavonoids (quercetin, hyperoside, rutin, amentoflavone) (Greeson et al., 2001). Traditionally, commercially available herbal preparations of this plant are standardized to hypericin (Baureithel et al., 1997), as it was initially regarded as the active principle in St. John's Wort extracts (Muldner and Zoller, 1984). This could not be confirmed in subsequent studies, however, and the constituents responsible for the effects of this antidepressant remain to be elucidated (Baureithel et al., 1997; von Eggelkraut-Gotlanka et al., 2002).

The flavonoids constitute a substantial amount of the biologically active compounds found in St. John's Wort, being found in concentrations as high as twelve percent of fresh plant, depending on the part of plant examined (Greeson et al., 2001). Indeed, flavonoid compounds are found in all vascular plants, and therefore constitute a major part of the human diet (Medina et al., 1997; Campbell et al., 2001; Marder and Paladini, 2002). Although the full extent of biological activity of these compounds is largely unknown, research into flavonoid actions on the central nervous system has begun to indicate effects at some neurotransmitter receptors (Butterweck et al., 2002). In particular, there has been increasing evidence that flavonoid effects may be mediated by the γ-aminobutyric acid$_A$ (GABA$_A$) receptor benzodiazepine ligand binding site (Nielsen et al., 1988; Medina et al., 1997; Marder and Paladini, 2002; Butterweck et al., 2002). Interestingly enough, amentoflavone, a biflavonoid constituent of St. John's Wort, was the first flavonoid compound to be shown to have high affinity for brain benzodiazepine receptors *in vitro*, being able to inhibit [^{3}H]-flunitrazepam binding with an IC$_{50}$ of 6 nM (see Fig. 3-1; Nielsen et al., 1988). Experiments with crude extracts of *Hypericum perforatum* also point to GABA$_A$ receptor involvement in the biological activity of its constituents. Using *in vitro* radioligand receptor binding assays, Cott (1997) showed that *Hypericum perforatum* extract had a high affinity for GABA$_A$ receptors. Behavioral studies with St. John's Wort have also implicated the involvement of the benzodiazepine binding site of the GABA$_A$ receptor in manifesting the effects of St. John's Wort. *Hypericum perforatum* total extract produced anxiolytic activity as measured by the light-dark test, and pretreatment of rats with flumazenil, a benzodiazepine site antagonist, abolished this effect (Vandenbogaerde et al., 2000). This finding is in agreement with clinical studies that have revealed a marked reduction in anxiety symptoms in depressive patients who were treated with St. John's Wort (Stevinson et al., 1998; Friede et al., 2001). Finally, a recent behavioral study where systematic removal of individual constituents from St. John's Wort extracts was performed concluded that *Hypericum perforatum* preparations devoid of hyperforin and hypericin, but enriched in flavonoids, still exerted antidepressant activity (Butterweck et al., 2003). This finding once again implicates the flavonoid class of compounds in the therapeutic efficacy of St. John's Wort.

Since the discovery that some flavonoids are competitive ligands at the GABA$_A$ receptor benzodiazepine binding site, research has focused on understanding the structure-activity relationship of these compounds at this receptor (Marder and Paladini, 2002). In addition to amentoflavone, a number of other flavonoids have been shown to have affinity, albeit lower than that of amentoflavone, for the benzodiazepine site via radioligand binding assays (Medina et al., 1997). These include both natural (apigenin, chrysin) and synthetic (6-bromo-3'-nitroflavone, 6,3'-dinitroflavone) flavonoids. *In vivo* experiments with chrysin, and to some extent apigenin, reveal anxiolytic activity, which in the case of chrysin can be abolished by prior administration of a benzodiazepine site antagonist (Marder and Paladini, 2002). These results also seem to correlate with the idea of $GABA_A$ receptor benzodiazepine site-mediated activity of this class of compounds.

Current investigations of flavonoids as allosteric modulators of $GABA_A$ receptors have focused on the benzodiazepine binding site. Recently, however, one group of researchers noted that the proposed benzodiazepine-like mechanism of action of flavonoids at $GABA_A$ receptors is based mainly on the correlation between affinity binding studies and *in vivo* pharmacological studies (Goutman et al., 2003). Their study set out to determine the modulation of GABA-gated chloride currents via electrophysiological experiments. The results of this study showed that in addition to inhibiting ionic currents mediated by the $GABA_A$ receptor, the group of natural and synthetic flavonoids studied is also capable of inhibiting ionic currents mediated by ρ_1 $GABA_C$ receptors. This is significant because $GABA_C$ receptors are normally insensitive to benzodiazepines. Also of note, the researchers observed that the strong inhibitory effects of apigenin and quercetin (natural flavonoids) on these currents could not be blocked by application of the benzodiazepine site antagonist flumazenil. Another study used functional $GABA_A$ receptor assays to show that a semisynthetic preparation of amentoflavone acted as a negative modulator of GABA at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, and that this negative modulation was not blocked by the addition of the benzodiazepine site antagonist flumazenil (Hanrahan et al., 2003). These findings are surprising in light of previous studies which seem to indicate a classical benzodiazepine-like mechanism of flavonoid modulation at the $GABA_A$ receptor. Although radioligand binding studies provide evidence that these compounds do indeed have affinity for the benzodiazepine binding site, functional studies now seem to suggest that the mechanism of action is more complex.

The present study examined the binding affinity of amentoflavone at recombinant wild type and mutant $GABA_A$ receptors. In particular, recombinant wild type receptors containing α_1, α_4, α_5, or α_6 subunits expressed in combination with β_2 and γ_2 subunits were investigated. Additionally, mutant receptors incorporating the array of histidine 101 mutant α_1 subunits constructed previously in this laboratory were also studied (Davies et al., 1998). Changes in affinity resulting from amino acid substitution at histidine 101, as compared to rat brain membrane $GABA_A$ receptors and wild type recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, were examined. Functional modulation of GABA-gated chloride currents, measured from recombinant wild type and mutant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes was assessed using the two-electrode voltage-clamp technique. Results suggest that the biflavonoid amentoflavone binds to recombinant wild type $GABA_A$ receptors expressing different α subunits in a manner resembling that of the classical benzodiazepine agonist diazepam (Fig. 3-1). Despite the high affinity of amentoflavone for the $GABA_A$ receptor, and the observation that the pattern of binding is similar to that of diazepam, negative modulation of GABA-gated chloride flux of $GABA_A$ receptors by this biflavonoid may occur by means of a mechanism independent of both the benzodiazepine and loreclezole sites. Results of this work will add to the knowledge of allosteric modulation of the $GABA_A$ receptor, and increase understanding with regard to the role that flavonoid compounds may play in the therapeutic efficacy of St. John's Wort.

2. Results

2.1 Amentoflavone binding at $GABA_A$ receptors containing different α subunits

The binding affinity of amentoflavone for recombinant wild type $GABA_A$ receptors was examined. Competition binding experiments were carried out using membranes from tsA201 cells, where α_1, α_4, α_5, or α_6 $GABA_A$ receptor subunits had been transiently expressed in combination with β_2 and γ_2 subunits . $[^3H]$Ro15-4513, a benzodiazepine site inverse agonist that binds to each of these $GABA_A$ receptor subtypes with nanomolar affinity, was used as the radioligand. Amentoflavone was able to displace bound $[^3H]$Ro15-4513 from all of the recombinant preparations with nanomolar affinity, except from the $\alpha_4\beta_2\gamma_2$ and $\alpha_6\beta_2\gamma_2$ receptor subtypes (Fig. 3-2). A slight increase in radioligand binding was noted for the $\alpha_5\beta_2\gamma_2$ receptor subtype at low concentrations of amentoflavone as compared to $\alpha_1\beta_2\gamma_2$; however K_i values were similar between the two subtypes. Amentoflavone also displaced $[^3H]$Ro15-1788 in competition experiments using rat brain membrane preparations. The affinities of amentoflavone for rat brain membrane and recombinant $GABA_A$ receptor subtypes are summarized in Table 3-1.

2.2 Amentoflavone binding at histidine 101 mutant $GABA_A$ receptors

Site-directed mutagenesis was performed previously to incorporate different amino acid substitutions at the histidine 101 site of the rat α_1 subunit (Davies et al., 1998). Four of these substitutions were examined in the present study. Amentoflavone displaced [^{3}H]Ro15-4513 at receptors containing the glutamine and tyrosine mutations, with only slight decreases in affinity observed as compared to recombinant wild type $\alpha_1\beta_2\gamma_2$ receptors (Fig. 3-3; Table 3-2). A complete loss of binding by amentoflavone was revealed for receptors containing the lysine and alanine mutations, as no significant displacement of bound [^{3}H]Ro15-4513 was observed.

2.3 Functional effects of amentoflavone at $GABA_A$ receptor subtypes

Two-electrode voltage clamp electrophysiology was used to determine functional effects of amentoflavone at recombinant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. No effect was observed when amentoflavone was tested alone, at nanomolar and micromolar concentrations, with oocytes expressing recombinant $\alpha_1\beta_2\gamma_2$ wild type $GABA_A$ receptors. In contrast, amentoflavone displayed a significant negative modulation of GABA-evoked currents at these receptors, and was able to inhibit the EC_{50} GABA response almost completely (IC_{50} = 2.23μM, 95% CI: 0.17-29) (Fig. 3-4). Amentoflavone showed a comparable negative modulation of GABA-evoked currents at recombinant $\alpha_1\beta_2$ wild type $GABA_A$ receptors (IC_{50} = 1.20μM, 95% CI: 0.75-1.9) (Fig. 3-4B). This receptor subtype is normally insensitive to classical benzodiazepine modulation, as it lacks the required γ subunit. No effect was seen when amentoflavone was tested alone at the $\alpha_1\beta_2$ $GABA_A$ receptor subtype.

A negative modulation of GABA-evoked currents at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors containing the alanine mutation at position 101 of the α_1 subunit, expressed in oocytes, was observed to be comparable to that seen at recombinant wild type $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, (IC_{50} = 1.10μM, 95% CI: 0.25-4.9) (Fig. 3-4B). Similar to the other $GABA_A$ receptor subtypes tested (above), amentoflavone produced no observable effects when given alone. Figure 3-4B illustrates the inhibitory effect of amentoflavone on the EC_{50} GABA response of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype containing the alanine mutation. When data were analyzed using one-site binding analysis, the data appeared biphasic; a significant difference was found with regard to curve fit when two-site binding analysis of the same data was performed. Data were therefore presented using two-site binding analysis, as a biphasic effect was apparent.

Recombinant $\alpha_1\beta_1\gamma_2$ GABA$_A$ receptors, expressed in oocytes, were tested to determine whether a β_1 versus β_2 subunit effect of amentoflavone could be established, similar to that seen for loreclezole and the β-carbolines (Stevenson et al., 1995). Amentoflavone given alone produced no effect, but showed a significant negative modulation of the EC$_{50}$ GABA response at this receptor subtype (Fig. 3-4B), similar to that observed for recombinant $\alpha_1\beta_2\gamma_2$ GABA$_A$ receptors (IC$_{50}$ = 0.764μM, 95% CI: 0.028-21). All electrophysiology data for amentoflavone are summarized in Table 3-3.

Amentoflavone was tested at a nanomolar concentration to determine whether the functional effects of the classical benzodiazepine positive modulator diazepam at recombinant $\alpha_1\beta_2\gamma_2$ GABA$_A$ receptors expressed in oocytes could be blocked. Positive modulation of GABA-evoked current by diazepam at this receptor subtype was inhibited by approximately one-half upon the addition of an equal concentration of amentoflavone (Fig. 3-5). Amentoflavone was also tested at 5HT$_3$ receptor subtypes. Preliminary data suggest that amentoflavone given alone produces no effect, but at micromolar concentrations is able to inhibit 5HT-evoked currents at both homomeric 5HT$_{3A}$ (Fig. 3-6A) and heteromeric 5HT$_{3AB}$ receptors (Fig. 3-6B).

3. Discussion

3.1 Amentoflavone and diazepam binding at the GABA$_A$ receptor

In the present study, amentoflavone binding was shown to differentiate between GABA$_A$ receptor subtypes containing different α subunits in a manner resembling diazepam binding. Although amentoflavone showed affinity for subtypes containing the α_1 or α_5 subunit, no binding was observed at GABA$_A$ receptor subtypes containing the α_4 or α_6 subunit. This suggests that amentoflavone binding at the GABA$_A$ receptor may involve binding contacts that also play a role in the binding of classical benzodiazepine positive modulators like diazepam (Fig. 3-1). A slight increase in radioligand binding was noted for the $\alpha_5\beta_2\gamma_2$ receptor subtype at low concentrations of amentoflavone as compared to $\alpha_1\beta_2\gamma_2$; however the reason for this is unclear. It has been suggested that one of the lipophilic pockets of the benzodiazepine site of the $\alpha_5\beta_2\gamma_2$ GABA$_A$ receptor subtype is larger in size than the analogous region in other receptor subtypes, and that occupation of this region may be important to high affinity biflavonoid binding (Marder et al., 2001). It is possible that amentoflavone interaction with this particular subtype may be leading to a potentiation of the interaction of other benzodiazepine site ligands such as Ro15-4513, the radioligand used in these experiments. It is difficult to speculate, however, the exact mechanism by which such an increase in interaction might occur and it cannot be ruled out that the increase in radioligand binding seen at the $\alpha_5\beta_2\gamma_2$ receptor subtype at low concentrations of amentoflavone in this study may simply be an experimental artifact. Nonetheless, K_i values for amentoflavone were found to be similar between the $\alpha_5\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_2$ subtypes, allowing for a comparison of DS versus DI GABA$_A$ receptor subtypes with regard to amentoflavone affinity.

Further evidence supporting a similar manner of binding for amentoflavone and diazepam at the benzodiazepine site involves histidine 101 (rat α_1 subunit). As mentioned, the presence of a histidine or arginine residue at this position has been shown to be responsible for the diazepam-sensitive and diazepam-insensitive GABA$_A$ receptor subtypes, respectively (Wieland et al., 1992). In addition, studies involving histidine 101 mutant GABA$_A$ receptors have supported the importance of this residue to the binding and function of classical benzodiazepine positive modulators such as flunitrazepam, as well as other benzodiazepine site ligands such as Ro15-4513 and Ro15-1788 (Davies et al., 1998; Dunn et al., 1999). In this study, amentoflavone displacement of the radioligand was abolished by mutation of histidine 101 to either an alanine or lysine residue. This finding suggests that histidine 101 plays an important role in the ability of amentoflavone to bind to the GABA$_A$ receptor. Differences in the structure of the residue at position 101 may be responsible for the effect seen on amentoflavone binding, albeit this is difficult to conclude (Fig. 3-7). When histidine 101 is mutated, there is a loss of its imidazole side moiety. However, comparison of the side chains of the replacement residues studied provides no obvious reason for the loss of affinity for amentoflavone at two of the mutant receptors. Tyrosine and glutamine both contain side chains which are uncharged polar groups, whereas alanine and lysine have a nonpolar methyl group side chain and a positively charged polar butylammonium side chain, respectively. Interestingly, the lysine mutation was also shown to result in a complete loss of classical benzodiazepine positive modulator flunitrazepam binding at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, whereas the tyrosine and glutamine mutations only resulted in right-fold shifts in flunitrazepam binding affinity (Dunn et al., 1999). In addition, the small size of the methyl group side chain of alanine may play a role in the loss of amentoflavone affinity observed with that mutation. Therefore, the possibility that changes in steric or charge-charge interactions are involved in the dramatic changes in affinity for amentoflavone, seen with the mutant receptors containing the alanine or lysine residue, cannot be excluded.

3.2 Functional effects of amentoflavone distinct from benzodiazepine site binding

Previous work with amentoflavone has shown that it acts as a negative modulator of GABA-gated chloride currents at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Hanrahan et al., 2003). In agreement with this finding, functional analysis of amentoflavone in the current study also revealed that it acts as a negative modulator of chloride currents elicited by an EC_{50} concentration of GABA at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (IC_{50} = 2.23μM, 95% CI: 0.17-29). However, it appears that these functional effects of amentoflavone may not be mediated through the same site at which high affinity binding occurs. The functional effects of amentoflavone at this $GABA_A$ receptor subtype were observed at micromolar concentrations, whereas radioligand binding experiments showed that amentoflavone binds to $GABA_A$ receptor with nanomolar affinity (Table 3-3; Table 3-1). In addition, it was observed that amentoflavone negatively modulated EC_{50} GABA-gated chloride currents at recombinant $\alpha_1\beta_2$ $GABA_A$ receptors (IC_{50} = 1.20μM, 95% CI: 0.75-1.9). These receptors do not contain a classical benzodiazepine binding site as they lack the proposed α-γ interface. The inhibition by amentoflavone observed at these receptors was not significantly different from that seen at the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype. This finding is in agreement with recent studies involving other flavonoids. Goutman et al. (2003) performed the first functional experiments that directly tested the effects of flavonoid compounds on receptor-mediated ionic currents. Their findings questioned whether flavonoid actions at $GABA_A$ receptors are actually mediated by the benzodiazepine binding site, despite the strong evidence that flavonoids do competitively bind to this site (Nielsen et al., 1988; Viola et al., 1995; Baureithel et al., 1997; Medina et al., 1997; Marder and Paladini, 2002). In addition to showing that flavonoids had similar actions at ρ_1 $GABA_C$ receptors, which lack the classical benzodiazepine site, they demonstrated that specific flavonoid actions at the $GABA_A$ receptor were flumazenil-insensitive. More recently, it was shown that 6-methylflavone has actions at $\alpha_1\beta_2$ $GABA_A$ receptors (Hall et al., 2004). Therefore, the observation that amentoflavone functionally modulates GABA-gated chloride currents at both recombinant $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ $GABA_A$ receptor subtypes in a similar manner, concurs with previous speculation that flavonoid actions at $GABA_A$ receptors may be mediated by a site different from that which mediates classical benzodiazepine modulation (Goutman et al., 2003; Hanrahan et al., 2003; Hall et al., 2004).

Functional analysis of the effects of amentoflavone at the mutant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype containing the alanine substitution at position 101 further substantiates the idea that flavonoids are acting at the $GABA_A$ receptor via a non-benzodiazepine site-mediated mechanism. Despite the complete abolishment of $GABA_A$ receptor binding of amentoflavone observed at this mutant receptor subtype (Fig. 3-3), amentoflavone displayed a functional effect at the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor containing the alanine mutation comparable to that seen at the wild type recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor. Although amentoflavone produced no effect when given alone, it was shown to be a negative modulator of GABA-gated chloride currents, with no significant difference between its inhibitory activity at the mutant receptor ($IC_{50}1$, Table 3-3) compared to the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype. This result suggests that even though high affinity binding at the $GABA_A$ receptor is lost, amentoflavone retains its functional activity at the mutant $GABA_A$ receptor by means of a different mechanism. Radioligand binding techniques used in the present study did not allow for the detection of the low affinity binding site(s) for amentoflavone, as a rapid filtration system is required to measure equilibrium binding parameters for faster dissociating sites. Nevertheless, the findings from the two-electrode voltage clamp experiments do suggest that the functional effects of amentoflavone at the mutant $GABA_A$ receptor are mediated by one or more lower affinity sites that are distinct from the benzodiazepine binding site.

Taken together, these data implicate a flavonoid mechanism of action at $GABA_A$ receptors that does not involve the classical benzodiazepine binding site. To further characterize amentoflavone interaction at the $GABA_A$ receptor, this study focused on the β subunit. The loreclezole site at the $GABA_A$ receptor is dependent upon the presence of a β_2 or β_3 subunit, and this site has also been shown to be a low affinity binding site for the β-carbolines (Wafford et al., 1994; Wingrove et al., 1994; Stevenson et al., 1995). A number of the β-carbolines are known to bind to the benzodiazepine site with high affinity, whereas low affinity binding to the loreclezole site was shown to discriminate between β_1- versus β_2- or β_3-containing $GABA_A$ receptors based on a single amino acid difference (Stevenson et al., 1995). It was observed that β-carboline low affinity binding to the loreclezole site was dependent upon the presence of an asparagine residue (β_2 and β_3; serine in β_1) in the transmembrane 2 region of the β subunit. Amentoflavone was tested at recombinant $\alpha_1\beta_1\gamma_2$ $GABA_A$ receptors to determine whether its effects at $GABA_A$ receptors are dependent upon the β subunit variant. No significant difference in the effects of amentoflavone at the $\alpha_1\beta_1\gamma_2$ $GABA_A$ receptor subtype was found compared to the wild type recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype. That is, amentoflavone negatively modulated GABA-gated chloride currents in a similar manner (IC_{50} = 0.764μM, 95% CI: 0.028-21), but had no effect when given alone. This suggests that amentoflavone does not discriminate between β1- and β2-containing $GABA_A$ receptor subtypes, and therefore is likely not acting via the loreclezole site.

Given that the functional effects of amentoflavone at the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype were observed at micromolar concentrations, whereas radioligand binding experiments showed that amentoflavone binds to $GABA_A$ receptor subtypes with nanomolar affinity, it was examined whether the modulation of GABA-evoked current by the classical benzodiazepine positive modulator diazepam could be blocked by the addition of a nanomolar concentration of amentoflavone. It was observed that the positive modulation of GABA-evoked current by diazepam was inhibited by approximately one-half upon the addition of an equal concentration of amentoflavone (Fig. 3-5). This observation supports findings from both the current study and previous studies (Nielsen et al., 1988; Baureithel et al., 1997) which suggest that amentoflavone does bind to the classical benzodiazepine binding site with high affinity, and further reveals that amentoflavone is acting as an antagonist.

In order to clarify whether the actions of amentoflavone are specific to the GABA_A receptor, amentoflavone was tested at 5HT_3 receptor subtypes, which are also members of the superfamily of ligand-gated ion channels. Amentoflavone applied alone produced no response; however preliminary data indicate that amentoflavone is able to inhibit 5HT-evoked currents at both the homomeric $\text{5HT}_{3\text{A}}$ and heteromeric $\text{5HT}_{3\text{AB}}$ receptor subtypes (Fig. 3-6). This inhibition was observed at micromolar concentrations of amentoflavone, similar to observations made at the GABA_A receptor. It is possible then that the observed functional effects of amentoflavone may be mediated by a non-specific mechanism common to both of these ligand-gated ion channel receptor types. One possibility is that the lack of intrinsic efficacy observed when this compound is applied alone at these receptors as opposed to the negative modulatory effect observed in the presence of agonist is indicative of use-dependent block as a mechanism for the inhibition of current in the presence of amentoflavone. Nevertheless, the idea cannot be excluded that these functional effects of amentoflavone may be mediated via completely different mechanisms at the GABA_A and 5HT_3 receptors respectively, albeit with similar inhibitory activity observed at subtypes of each. More research is required to further elucidate the exact manner by which amentoflavone interacts with each of these receptors.

Numerous studies have been undertaken to create and refine a model of flavonoid binding at the benzodiazepine binding site (Dekermendjian et al., 1999; Marder et al., 2001; Kahnberg et al., 2002; Hong and Hopfinger, 2003). In addition, attempts have been made to ascertain specific substituents that may increase the affinity of flavonoid binding to this site (Huen et al., 2003). Of both naturally-occurring and synthetic flavonoids, amentoflavone has one of the highest affinities for the benzodiazepine site (Medina et al., 1997; Marder and Paladini, 2002). Amentoflavone, or I3, II8-biapigenin, is biflavonoid in structure, with its half-structure being the structure of the flavonoid apigenin. Apigenin has also been shown to bind at the benzodiazepine site, albeit with much less affinity than amentoflavone (Viola et al., 1995). Functional studies performed using apigenin have shown that it has a very modest negative modulatory effect on

GABA-induced chloride currents at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Goutman et al., 2003; Campbell et al., 2004). In contrast, the same concentration of amentoflavone has a considerably higher efficacy at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. This difference may be attributable to amentoflavone being a much larger and/or more hydrophobic molecule than apigenin, being twice its size. These may be the reasons for an enhancement of both benzodiazepine site- and non-benzodiazepine site-mediated interactions of amentoflavone at the $GABA_A$ receptor, in comparison to apigenin. Interestingly, a recent study fitted both apigenin and amentoflavone into a 3D-pharmacophore model for the benzodiazepine receptor (Svenningsen et al., 2006). It was concluded that while apigenin and one of the flavone backbones of amentoflavone fit the model similar to other flavone derivatives, the second flavone backbone of amentoflavone could additionally be fitted into a "subunit interface" area of the model that was likely resulting in affinity increasing interactions as compared to apigenin, thus leading to higher affinity of the biflavonoid for the receptor. The proposed "subunit interface" area is suggested to correspond to the α-γ subunit interface of the $GABA_A$ receptor. Alternately, the idea of use-dependent block as a mechanism by which amentoflavone negatively modulates the GABA-gated chloride current might suggest that the biflavonoid structure of this compound could be amenable to one of the flavone backbones acting as a channel-blocker upon agonist-activated channel opening. While this proposed mechanism has not yet been investigated using molecular modeling, it should be regarded as a possible means by which the biflavonoid amentoflavone displays a greater negative modulatory effect than the related monoflavonoid compound apigenin. Additional binding and functional experiments combined with more extensive modeling studies will hopefully permit better elucidation of the undoubtedly complex mechanisms by which amentoflavone interacts with the $GABA_A$ receptor.

These results suggest that amentoflavone interactions at $GABA_A$ receptors include binding to high affinity binding sites, as well as functional effects not mediated by these binding sites. Interestingly, this biflavonoid binds to $GABA_A$ receptor subtypes in a manner resembling diazepam binding, and similar to diazepam, amentoflavone affinity for the $GABA_A$ receptor is affected by changes to histidine 101. Nevertheless, these observations suggest that amentoflavone is acting as an antagonist at the high affinity site, and that the functional effects at $GABA_A$ receptors are most likely mediated by mechanisms distinct from the benzodiazepine and loreclezole binding sites.

With regard to St. John's Wort therapy, the role of amentoflavone in the efficacy of this herbal remedy as a treatment for depression and the anxiety symptoms associated with this condition remains unclear. While it is likely that a number of different compounds contribute as active constituents in the herbal extracts of this plant (Greeson et al., 2001), it is possible that amentoflavone, shown in the present study to have significant effects at ligand-gated ion channel receptors, could affect the overall efficacy of St. John's Wort preparations. The negative modulatory effects of micromolar concentrations of this biflavonoid compound at the $GABA_A$ receptor seem counterintuitive to the anxiolytic actions of classical benzodiazepines, which are associated with positive modulation of GABA-induced chloride current, and it cannot be ruled out that the presence of amentoflavone within preparations of St. John's Wort may actually decrease the overall efficacy of this herbal remedy. Nevertheless, the experimental results seen within this study which show that nanomolar concentrations of amentoflavone have effects at the $GABA_A$ receptor, including displacement of benzodiazepine-site ligand binding and the inhibition of diazepam-mediated potentiation of GABA-gated chloride current, could indicate more complex actions of amentoflavone in regards to behavioral effects. For instance, the presence of small concentrations of amentoflavone within the brain which affect the function of $GABA_A$ receptor subtypes may ultimately lead to downstream effects that alter the balance of excitation and inhibition and, as such, play a role in mediating antidepressant and/or anxiolytic effects. A recent *in vitro* study which outlines the transport of amentoflavone across the blood-brain barrier via passive diffusion further supports the idea that the presence of this biflavonoid molecule in St. John's Wort extracts could affect the overall activity of the herbal remedy in the central nervous system (Gutmann, et al., 2002). Additionally, the observed effects of amentoflavone at $5HT_3$ receptor subtypes also indicate that this biflavonoid may interact within the brain via complex mechanisms. As $5HT_3$-mediated mechanisms have been implicated in antidepressant and anxiolytic therapies (Dremencov et al., 2006; Harmer et al., 2006), more research into amentoflavone action at these receptor subtypes is warranted to determine whether the efficacy of St. John's Wort could in part be due to this interaction. Ultimately, further research is required to elucidate the complete role of amentoflavone, and flavonoids in general, within plant preparations in order to determine whether the effectiveness of natural remedies such as St. John's Wort can be attributed, at least in part, to the action of these compounds.

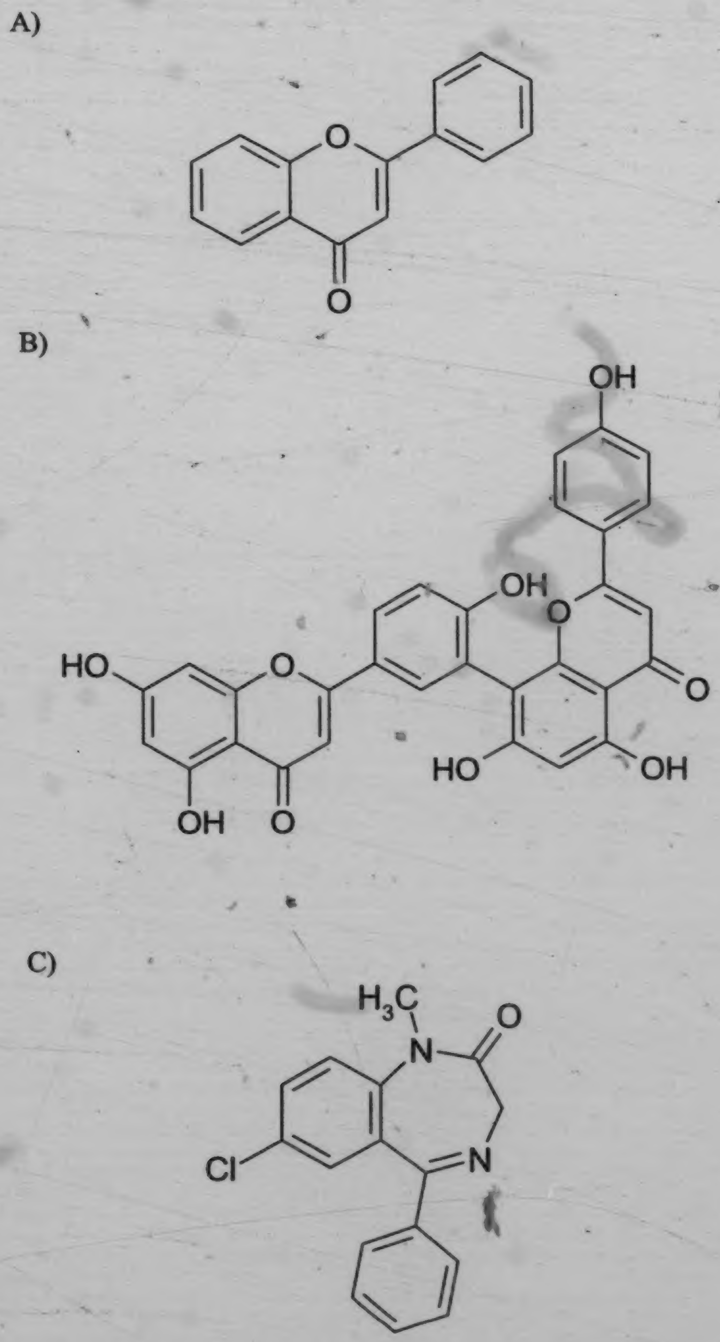

Figure 3-1

Chemical structures of (A) flavone, (B) amentoflavone (a biflavonoid compound), and (C) diazepam are depicted for comparison.

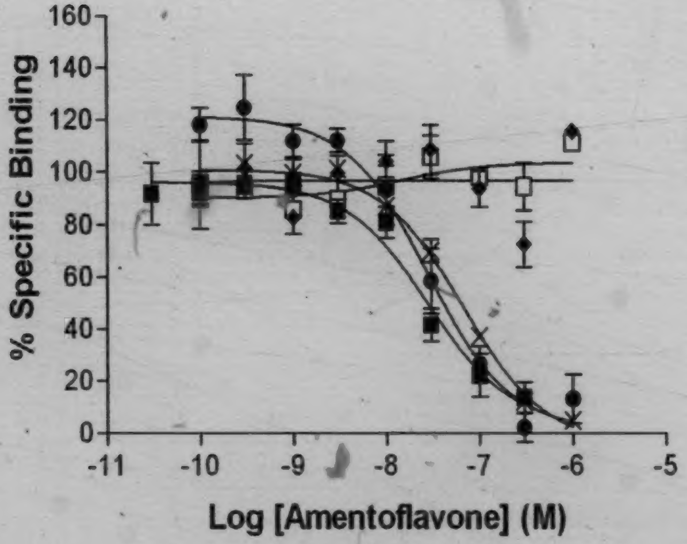

Figure 3-2

Displacement of $[^3H]$Ro15-4513 by amentoflavone at $GABA_A$ receptors containing different α subunits. Competition binding curves using cell membrane expressing $\alpha_1\beta_2\gamma_2$ (■), $\alpha_4\beta_2\gamma_2$ (◆), $\alpha_5\beta_2\gamma_2$ (●), and $\alpha_6\beta_2\gamma_2$ (□) $GABA_A$ receptor subtypes. Rat brain membrane (×) is also included for comparison. $[^3H]$Ro15-4513 was present at a concentration equal to its K_d value as determined by saturation analysis. For competition assays using rat brain membrane, $[^3H]$Ro15-1788 was used as the radioligand. Data are from at least three separate experiments performed in duplicate. Results represent mean ± SEM.

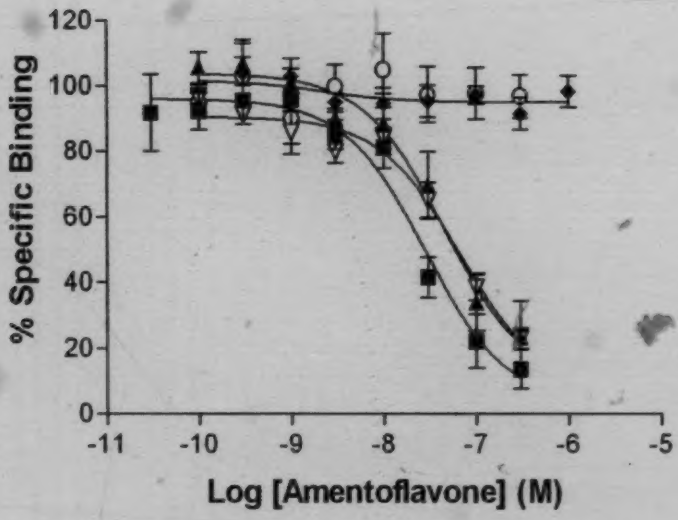

Figure 3-3

Displacement of [^{3}H]Ro15-4513 by amentoflavone at GABA$_A$ receptor histidine 101 mutants. Competition binding curves using cell membrane expressing glutamine (▲), tyrosine (▽), lysine (◆), and alanine (○) mutations at position 101 of the α_1 subunit are shown. $\alpha_1\beta_2\gamma_2$ (■) is also included for comparison. [^{3}H]Ro15-4513 was present at a concentration equal to its K_d value as determined by saturation analysis. Data are from at least three separate experiments performed in duplicate. Results represent mean ± SEM.

A)

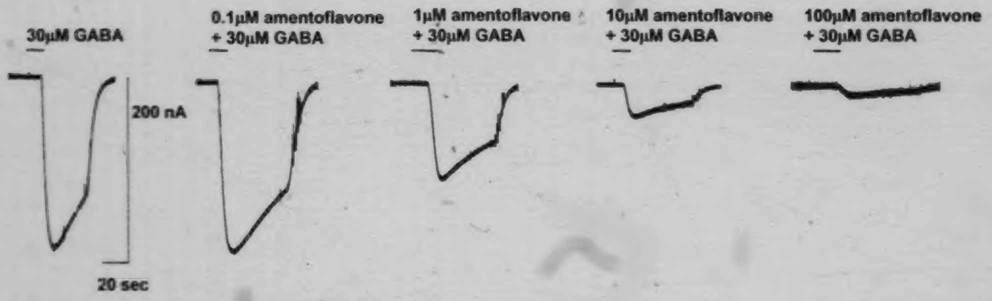

Figure 3-4

Negative modulation of GABA-mediated currents by amentoflavone.

(A) Current traces recorded when amentoflavone was tested with oocytes expressing recombinant $\alpha_1\beta_2\gamma_2$ wild type $GABA_A$ receptors. A 3 min perfusion of each amentoflavone concentration (not shown) was followed by coapplication of amentoflavone with an EC_{50} concentration of GABA.

B)

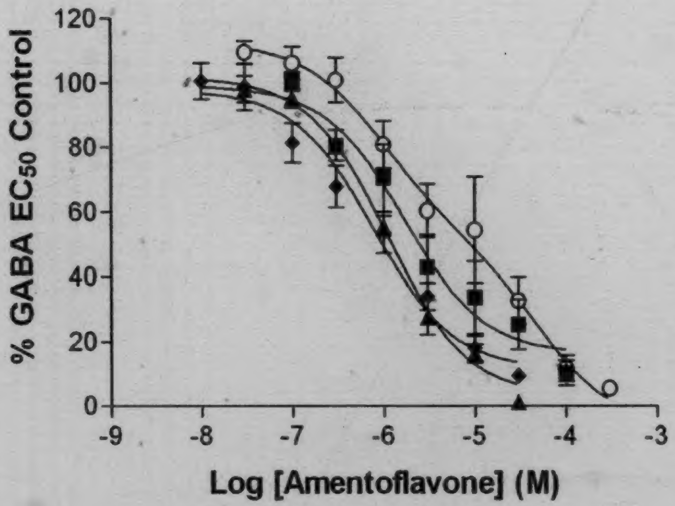

Figure 3-4

Negative modulation of GABA-mediated currents by amentoflavone.

(B) The effects of amentoflavone on currents induced by GABA at a concentration approximately equal to its EC$_{50}$ value in the $\alpha_1\beta_2\gamma_2$ (■), $\alpha_1\beta_2$ (▲), $\alpha_1\beta_2\gamma_2$ containing the alanine mutation (○), and $\alpha_1\beta_1\gamma_2$ (◆) receptors are shown. Data are from observations made from at least three oocytes. Results represent mean ± SEM.

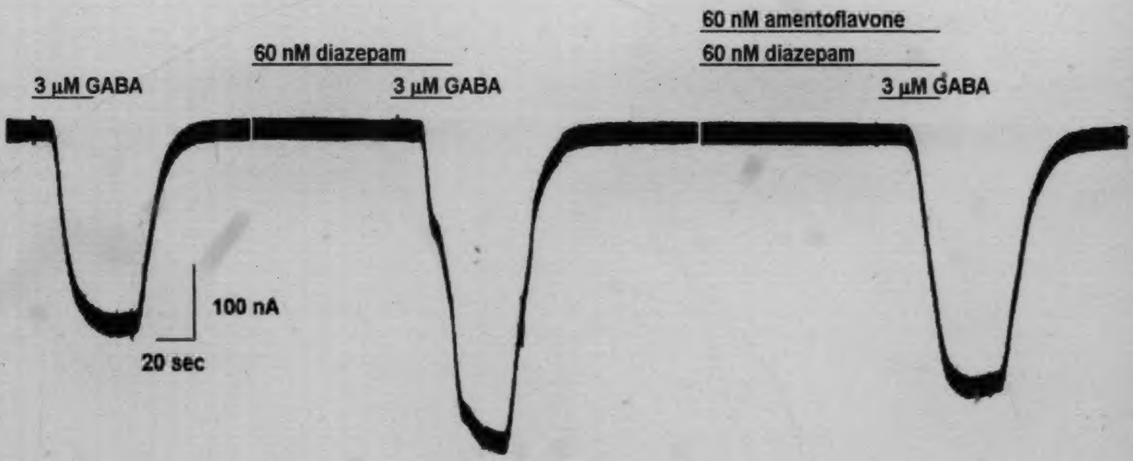

Figure 3-5

Potentiation of the GABA-mediated currents at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors by diazepam is inhibited by an equal concentration of amentoflavone. Current traces recorded from an oocyte expressing recombinant $\alpha_1\beta_2\gamma_2$ wild type $GABA_A$ receptors in response to 3µM GABA are shown. Potentiation of the GABA-mediated current upon perfusion of 60nM diazepam followed by coapplication with 3µM GABA is shown, and inhibition of this potentiation with the addition of 60nM amentoflavone.

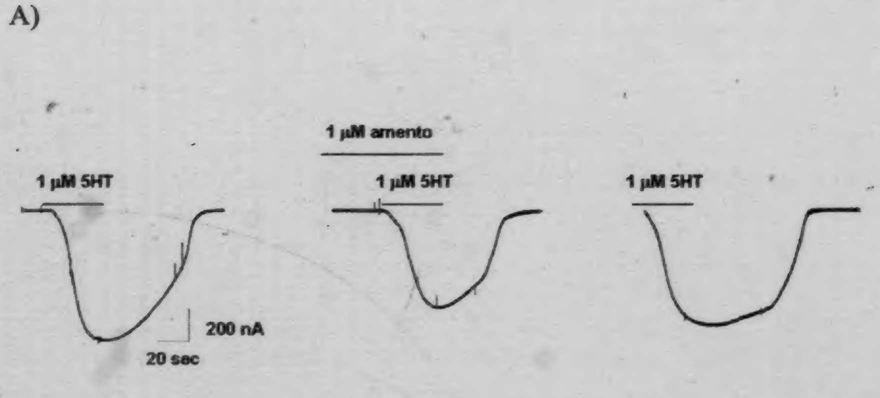

Figure 3-6

Negative modulation of 5HT-mediated currents by amentoflavone.

(A) Current traces recorded when amentoflavone was tested with oocytes expressing recombinant wild type $5HT_{3A}$ receptors. A 3 min perfusion of a 1µM amentoflavone concentration (not shown) was followed by coapplication of amentoflavone with an EC_{50} concentration of 5HT.

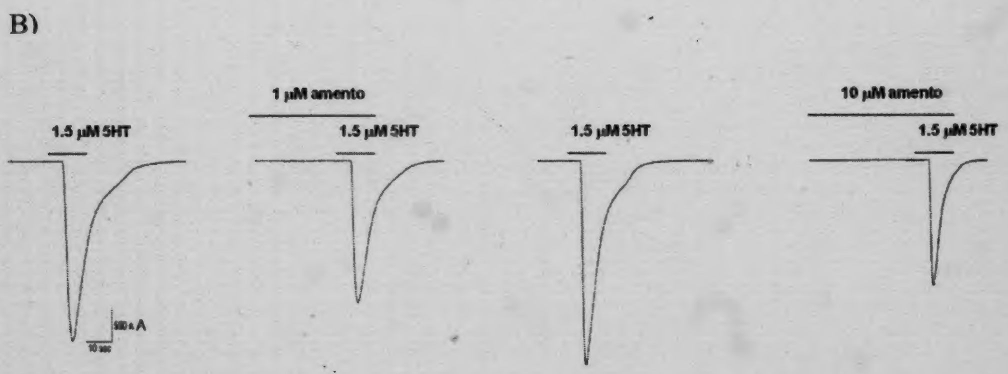

Figure 3-6

Negative modulation of 5HT-mediated currents by amentoflavone.

(B) Current traces recorded when amentoflavone was tested with oocytes expressing recombinant wild type $5HT_{3AB}$ receptors. A 3 min perfusion of 1µM and 10µM amentoflavone concentrations respectively (not shown) were followed by coapplication of amentoflavone with an EC_{50} concentration of 5HT.

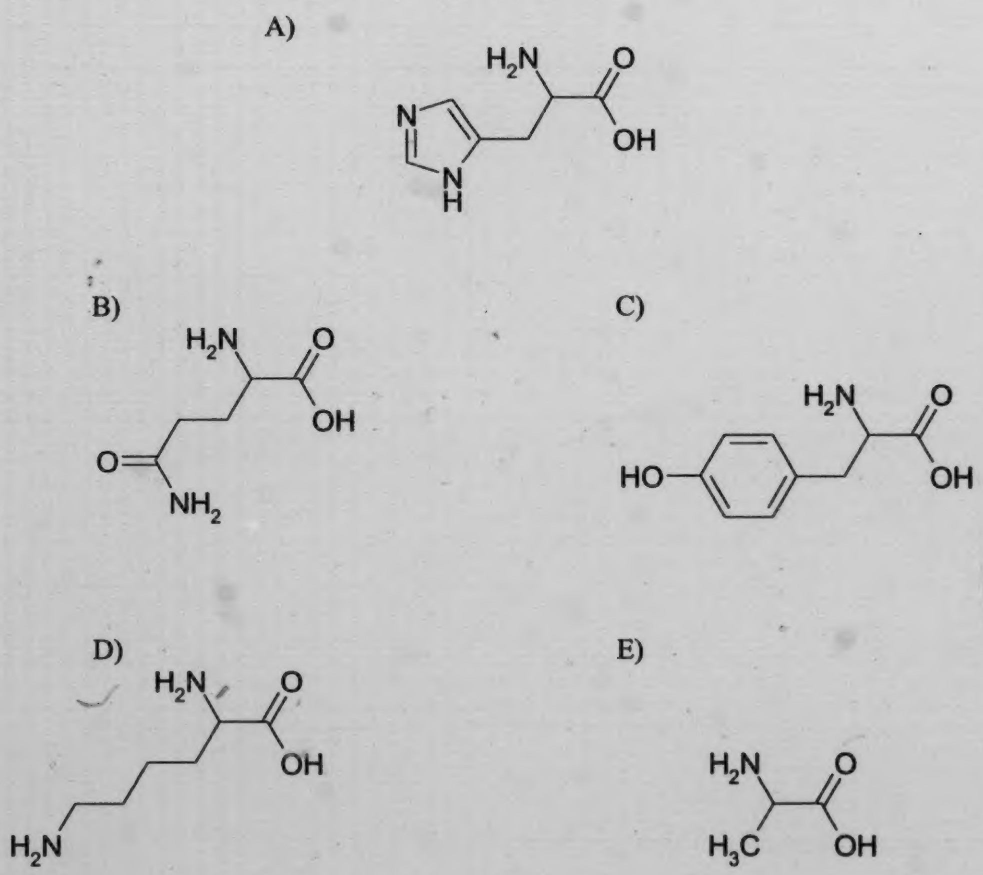

Figure 3-7

Chemical structures of the amino acid mutations examined in this chapter are depicted for comparison of their side chains. The structure of the histidine residue (A), normally present at position 101 of the α_1 subunit, is shown, as well as the structures of (B) glutamine, (C) tyrosine, (D) lysine, and (E) alanine, which were incorporated as mutations.

	Affinity	
α subunit	**Log IC_{50} ± SEM (M)**	**K_i (nM)**
$\alpha_1\beta_2\gamma_2$	-7.57 ± 0.1	13.8
$\alpha_4\beta_2\gamma_2$	N.D.	N.D.
$\alpha_5\beta_2\gamma_2$	-7.54 ± 0.1	15.0
$\alpha_6\beta_2\gamma_2$	N.D.	N.D.
rat[a]	-7.20 ± 0.06	23.9

Table 3-1

Effects of α subunit substitution on the affinity of amentoflavone for $GABA_A$ receptor subtypes. All values are derived from displacement of [^{3}H]Ro15-4513 at a concentration equal to its K_d value for each receptor subtype. N.D. indicates values not determined. Data are from at least three experiments performed in duplicate.

[a] Rat brain membrane competition assays were performed as described above using [^{3}H]Ro15-1788 as the radioligand.

	Affinity	
α_1 subunit mutation	**Log IC_{50} ± SEM (M)**	**K_i (nM)**
glutamine	-7.40 ± 0.2	19.9
tyrosine	-7.13 ± 0.2	30.6
lysine	N.D.	N.D.
alanine	N.D.	N.D.

Table 3-2

Effects of α_1 subunit histidine 101 mutation on the affinity of amentoflavone for the $GABA_A$ receptor. All values are derived from displacement of [^{3}H]Ro15-4513 at a concentration equal to its K_d value for each receptor subtype. N.D. indicates values not determined. Data are from at least three experiments performed in duplicate.

Subtype	Log IC_{50} ± SEM (M)	IC_{50} (95% CI) (μM)
$\alpha_1\beta_2\gamma_2$	-5.65 ± 0.4	2.23 (0.17-29)
$\alpha_1\beta_2$	-5.92 ± 0.05	1.20 (0.75-1.9)
$\alpha_1\beta_2\gamma_2$ (alanine mutation)		
(1)[a]	-5.96 ± 0.2	1.10 (0.25-4.9)
(2)	-3.55 ± 0.5	285 (1.9-42 000)
$\alpha_1\beta_1\gamma_2$	-6.12 ± 0.3	0.764 (0.028-21)

Table 3-3

Negative modulation of GABA-mediated currents by amentoflavone. Data are from observations made from at least three oocytes. Experiments were performed using GABA EC_{50} concentrations.

[a] Data recorded from the $\alpha_1\beta_2\gamma_2$ subtype containing the alanine mutation at position 101 of the α_1 subunit were analyzed using two-site binding analysis (see Results).

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CHAPTER 4

Investigation of the Recognition Properties of the High Affinity Synthetic Flavonoid Derivative 5′-Bromo-2′-hydroxy-6-methylflavone for Different GABA$_A$ Receptor Subtypes

1. Introduction

Studies have demonstrated that flavonoids, a group of compounds found in all vascular plants, have affinity for the $GABA_A$ receptor benzodiazepine binding site (Nielsen et al., 1988; Medina et al., 1989). Subsequent studies have confirmed these findings (Medina et al., 1997; Viola et al., 1999; Dekermendjian et al., 1999; Marder et al., 2001; Kahnberg et al., 2002; Marder and Paladini, 2002; Butterweck et al., 2002); however, the majority of these affinity studies, using both natural and synthetic flavonoids, have been performed on rat cortical membranes and, as such, it is difficult to elucidate whether $GABA_A$ receptor subtype specificity plays a role in flavonoid binding to the benzodiazepine binding site. Recently, this laboratory reported that radioligand binding studies show that amentoflavone, a biflavonoid constituent of St. John's Wort, binds to recombinant wild type $GABA_A$ receptors expressing different α subunits in a manner resembling that of the classical benzodiazepine agonist diazepam (Hansen et al., 2005). Amentoflavone shows affinity for $GABA_A$ receptor subtypes containing the α_1 or α_5 subunit, but little or no affinity is observed for α_4- or α_6-containing $GABA_A$ receptors which have been classified as diazepam-insensitive $GABA_A$ receptor subtypes (Wieland et al., 1992). These findings suggest that in addition to binding to the benzodiazepine binding site, the flavonoids may in fact share binding contacts within the binding site cleft that are also involved in classical benzodiazepine agonist binding.

A number of studies have attempted to create a model of flavonoid binding at the benzodiazepine site, and to identify specific substituents on the basic flavone backbone structure that may increase the affinity of specific flavonoids for this site (Viola et al., 1999; Dekermendjian et al., 1999; Marder et al., 2001; Kahnberg et al., 2002; Huen et al., 2003; Hong and Hopfinger, 2004). In addition to investigating the properties of naturally occurring flavonoids, many synthetic derivatives have been created to further define the mechanism of binding. One such study involved the synthesis of 40 new flavone derivatives with subsequent determination of their affinities for the benzodiazepine site (Kahnberg et al., 2002). Many of these flavonoid derivatives were shown to have significant increases in affinity for the benzodiazepine site; these included 5′-bromo-2′-hydroxy-6-methylflavone, the highest affinity flavonoid derivative reported thus far, with a K_i value of 0.9 nM.

The present study further investigated the recognition properties of the high affinity flavonoid derivative 5′-bromo-2′-hydroxy-6-methylflavone for different $GABA_A$ receptor subtypes to determine whether this compound displays any subtype selectivity. Radioligand binding assays were used to examine the binding affinity of 5′-bromo-2′-hydroxy-6-methylflavone at recombinant wild type $GABA_A$ receptors containing α_1, α_2, α_4, α_5, or α_6 subunits expressed in combination with β_2 and γ_2 subunits. Affinity for a mutant $GABA_A$ receptor subtype incorporating a histidine to alanine amino acid substitution at position 101 of the α_1 subunit was also examined. Finally, functional effects of 5′-bromo-2′-hydroxy-6-methylflavone at recombinant wild type $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes were also measured using two-electrode voltage-clamp electrophysiology. Results suggest that, similar to the binding profile observed for the biflavonoid amentoflavone (Hansen et al., 2005), 5′-bromo-2′-hydroxy-6-methylflavone also binds to recombinant wild type $GABA_A$ receptors expressing different α subunits in a manner resembling that of the classical benzodiazepine agonist diazepam.

2. Results

2.1 5′-Bromo-2′-hydroxy-6-methylflavone binding at $GABA_A$ receptors containing different α subunits

The binding affinity of 5′-bromo-2′-hydroxy-6-methylflavone for recombinant wild type $GABA_A$ receptors containing different α subunits was examined by performing competition binding experiments. Membranes from tsA201 cells, where α_1, α_2, α_4, α_5, or α_6 $GABA_A$ receptor subunits had been transiently expressed in combination with β_2 and γ_2 subunits were used. [^{3}H]Ro15-4513 was used as the radioligand for these competition experiments, as this benzodiazepine site inverse agonist is known to bind to each of these $GABA_A$ receptor subtypes with nanomolar affinity. 5′-Bromo-2′-hydroxy-6-methylflavone displaced bound [^{3}H]Ro15-4513 from all of the recombinant preparations in a competitive manner with nanomolar affinity, with the exception of the $\alpha_4\beta_2\gamma_2$ and $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptor subtypes (Fig. 4-2). Competition experiments using rat brain membrane preparations also showed that 5′-bromo-2′-hydroxy-6-methylflavone was able to displace [^{3}H]Ro15-1788. Results demonstrating the affinity of 5′-bromo-2′-hydroxy-6-

methylflavone for rat brain membrane and recombinant $GABA_A$ receptor subtypes are summarized in Table 4-1.

2.2 5′-Bromo-2′-hydroxy-6-methylflavone binding at a histidine 101 mutant $GABA_A$ receptor

Site-directed mutagenesis was performed previously to incorporate different amino acid substitutions at the histidine 101 site of the rat α_1 subunit (Davies et al., 1998). The alanine substitution was examined in the present study. Preliminary data suggest a complete loss of binding by 5′-bromo-2′-hydroxy-6-methylflavone for $GABA_A$ receptors containing the alanine mutation, with no significant displacement of bound $[^3H]$Ro15-4513 seen (data not shown).

2.3 5′-Bromo-2′-hydroxy-6-methylflavone functional effects at recombinant wild type $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors

The functional effects of 5′-bromo-2′-hydroxy-6-methylflavone at recombinant wild type $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes were determined using two-electrode voltage-clamp electrophysiology. When tested alone, 5′-bromo-2′-hydroxy-6-methylflavone produced no observable effect. No change was observed for GABA-evoked currents at these receptors when 5′-bromo-2′-hydroxy-6-methylflavone was applied in conjunction with GABA. It was tested whether the positive modulation of GABA-evoked currents by the classical benzodiazepine positive modulator diazepam could be blocked by addition of 5′-bromo-2′-hydroxy-6-methylflavone. The positive modulation evoked by a nanomolar concentration of diazepam was shown to be inhibited by the addition of a low micromolar concentration of 5′-bromo-2′-hydroxy-6-methylflavone (Fig. 4-3).

3. Discussion

This laboratory previously demonstrated that amentoflavone, a biflavonoid compound, differentially binds to the benzodiazepine binding site of $GABA_A$ receptor subtypes in a manner resembling the classical benzodiazepine agonist diazepam (Hansen et al., 2005). The present study investigated the binding profile of 5′-bromo-2′-hydroxy-6-methylflavone at the benzodiazepine binding site of $GABA_A$ receptor subtypes containing different α subunits. 5′-Bromo-2′-hydroxy-6-methylflavone showed affinity for GABA$_A$ receptor subtypes containing the α_1, α_2, or α_5 subunit, whereas a loss of competitive binding was observed at GABA$_A$ receptor subtypes containing the α_4 or α_6 subunit. Similar to the findings for amentoflavone, these data suggest that 5′-bromo-2′-hydroxy-6-methylflavone may interact with the benzodiazepine binding site via binding contacts that are also involved in the binding of the classical benzodiazepine agonist diazepam (Fig. 4-1).

It has been shown that the amino acid residue present at position 101 of loop A (rat α_1 subunit) is important to the binding of classical benzodiazepine agonists such as diazepam (Wieland et al., 1992). Specifically, diazepam-sensitive and diazepam-insensitive classifications of GABA$_A$ receptor subtypes have been made based on the presence of a histidine or an arginine residue respectively. The α_4 and α_6 subunits contain the arginine residue at this position and therefore confer insensitivity to classical benzodiazepine agonists. This laboratory showed previously that binding of amentoflavone was abolished by the mutation of histidine 101 to an alanine residue (Hansen et al., 2005). Preliminary data from the present study suggest a complete loss of binding by 5′-bromo-2′-hydroxy-6-methylflavone for GABA$_A$ receptors containing the alanine mutation. This suggests that histidine 101 may play an important role in the binding of 5′-bromo-2′-hydroxy-6-methylflavone at the benzodiazepine site. Taken together with previous results seen for amentoflavone, these data may indicate that histidine 101, shown to be significant to the binding of classical benzodiazepine agonists, is important to the binding of flavonoids as well. Accordingly, mutation of this residue would result in changes in structure that might disrupt the ability of flavonoids to bind to the benzodiazepine site. Alternatively, it is not possible to exclude the possibility that the mutation of histidine 101 to an alanine residue specifically might result in dramatic changes to the structure of the benzodiazepine site that subsequently affects the ability of benzodiazepine site ligands in general to bind with high affinity. The methyl group side chain of alanine is small in size compared to the imidazole side moiety of histidine and therefore this particular mutation may be incompatible to overall ligand binding at the benzodiazepine site. Additional binding studies of flavonoids and other benzodiazepine site ligands at GABA$_A$ receptors containing the alanine mutation will be required to better elucidate the importance of histidine 101 to flavonoid binding in particular at the benzodiazepine site.

Functional experiments showed that 5′-bromo-2′-hydroxy-6-methylflavone produced no effect when applied alone at wild type $\alpha_1\beta_2\gamma_2$ GABA$_A$ receptors, nor produced any observable modulation of GABA-evoked currents. Given the aforementioned results of the radioligand binding studies that suggest 5′-bromo-2′-hydroxy-6-methylflavone binds to GABA$_A$ receptor subtypes in a manner resembling that of the classical benzodiazepine agonist diazepam, it was tested whether the positive modulation of GABA-evoked current by a nanomolar concentration of diazepam could be blocked by the addition of 5′-bromo-2′-hydroxy-6-methylflavone. The positive modulation of GABA-evoked current produced by diazepam was inhibited upon addition of 5′-bromo-2′-hydroxy-6-methylflavone (Fig. 4-3), suggesting that this flavonoid may be binding at the benzodiazepine binding site with high affinity and is likely acting as an antagonist.

Studies outlining proposed models of flavonoid binding to the benzodiazepine site have attempted to identify specific substituents on the flavone backbone that are essential to high affinity recognition (Dekermendjian et al., 1999; Viola et al., 1999; Marder et al., 2001; Kahnberg et al., 2002; Huen et al., 2003; Hong and Hopfinger, 2003). Principal observations have included the increase in benzodiazepine binding site affinity seen with 6,3′-disubstituted flavonoids and the significant increase in affinity observed with 2′-hydroxyl-substituted flavonoids. 5′-Bromo-2′-hydroxy-6-methylflavone contains substituents at the 6- and 3′(5′)-positions as well as the 2′-hydroxyl-substitution (Fig. 4-1), in accordance with the principal observations mentioned above, and it is therefore not surprising that this flavonoid is shown to have very high affinity for the benzodiazepine binding site. The majority of benzodiazepine binding site affinity studies investigating flavonoids have been performed using rat cortical membrane preparations and as such, differential binding of flavonoids to GABA$_A$ receptor subtypes has been difficult to ascertain. Findings of the present study suggest that 5′-bromo-2′-hydroxy-6-methylflavone binds to recombinant wild type GABA$_A$ receptors expressing different α subunits in a manner resembling diazepam. At present, it is only possible to speculate on the extent to which these findings can be applied to flavonoid binding at the benzodiazepine binding site in general. Amentoflavone does not contain the aforementioned 6,3′-disubstitution nor the 2′-hydroxyl-substitution, yet also displays differential affinity for the diazepam-sensitive $GABA_A$ receptor subtypes (Hansen et al., 2005), suggesting that substituents present elsewhere on the flavone backbone may be sufficient for high affinity benzodiazepine site binding. In contrast, the biflavonoid structure of amentoflavone may distinguish the requirements for high affinity binding at this site from that of monoflavonoids such as 5′-bromo-2′-hydroxy-6-methylflavone. It has been suggested that a number of flavonoids do not have affinity for the recombinant $\alpha_6\beta_3\gamma_2$ $GABA_A$ receptor subtype (Marder et al., 2001), a result that further substantiates the idea that the flavonoid class of compounds may altogether display a loss of affinity for diazepam-insensitive $GABA_A$ receptor subtypes. Alternatively, it has been suggested that K36, a naturally occurring 2'-hydroxyl-substituted flavonoid derivative, acts as a partial positive allosteric modulator at $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptors, a result which might indicate that some flavonoids do possess affinity for the benzodiazepine binding site at this $GABA_A$ receptor subtype (Huen et al., 2003). Nevertheless, this functional data might also be explained via mechanisms distinct from the classical benzodiazepine binding site, as such findings have also been reported for other flavonoids (Goutman et al., 2003; Hanrahan et al., 2003; Hall et al., 2004; Hansen et al., 2005), and therefore it cannot be ruled out that K36 might also display a lack of affinity for the benzodiazepine binding site at α6-containing $GABA_A$ receptor subtypes. Ultimately, further affinity studies are needed before it can be fully discriminated whether subtype-specific binding of flavonoids at the benzodiazepine binding site of $GABA_A$ receptors can be classified in a manner similar to classical benzodiazepines such as diazepam, based on commonalities in structure of the flavonoid class of compounds; or whether binding profiles at the benzodiazepine binding site of different $GABA_A$ receptor subtypes are specific to individual flavonoids based on specific differences in their structures.

In conclusion, these results reveal that 5′-bromo-2′-hydroxy-6-methylflavone binds to recombinant wild type $GABA_A$ receptors expressing different α subunits in a manner resembling that of the classical benzodiazepine agonist diazepam. Although preliminary data indicate that affinity of this monoflavonoid for the $GABA_A$ receptor is affected by changes to histidine 101, functional observations suggest that it is acting as an antagonist at this high affinity site. Additional affinity studies, using recombinant $GABA_A$ receptor subtypes, are required to determine whether the $GABA_A$ receptor subtype selective binding profile of 5′-bromo-2′-hydroxy-6-methylflavone, which parallels that seen for amentoflavone, can be extended to other flavonoids in general.

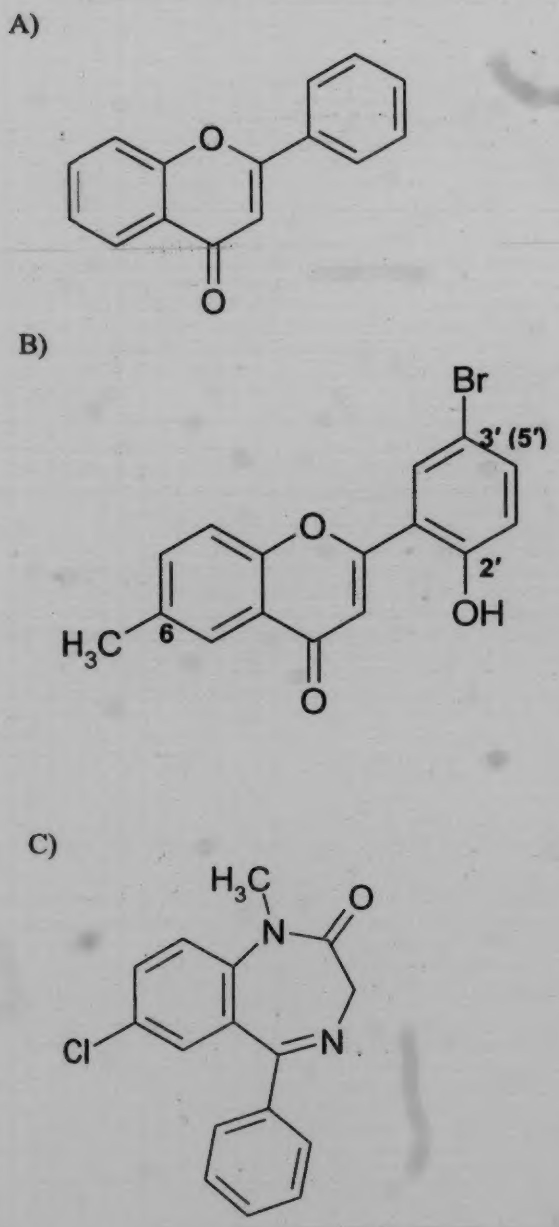

Figure 4-1

Chemical structures of (A) flavone, (B) 5′-bromo-2′-hydroxy-6-methylflavone, and (C) diazepam are depicted for comparison. Numbering of positions on the chemical backbone of 5′-bromo-2′-hydroxy-6-methylflavone is shown to indicate the position of substituent groups that have been associated with high affinity flavonoid binding at the benzodiazepine site (see Discussion).

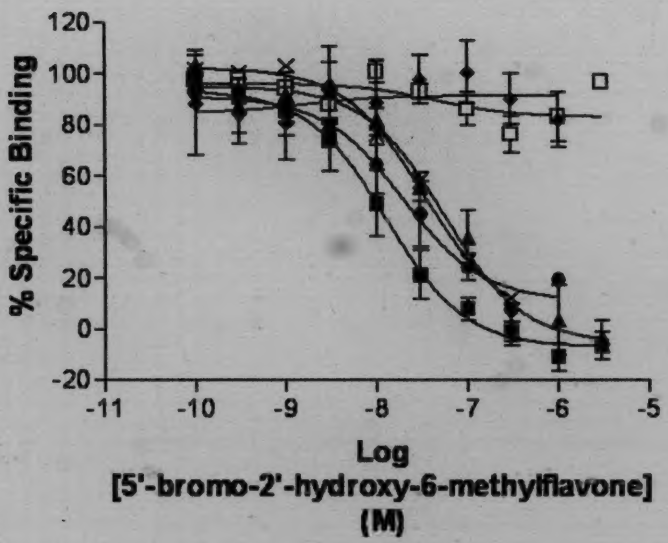

Figure 4-2

Displacement of [^{3}H]Ro15-4513 by 5′-bromo-2′-hydroxy-6-methylflavone at GABA$_A$ receptors containing different α subunits. Competition binding curves using cell membrane expressing $\alpha_1\beta_2\gamma_2$ (■), $\alpha_2\beta_2\gamma_2$ (▲), $\alpha_4\beta_2\gamma_2$ (♦), $\alpha_5\beta_2\gamma_2$ (●), and $\alpha_6\beta_2\gamma_2$ (□) GABA$_A$ receptor subtypes. Preliminary data using rat brain membrane (×) are also included for comparison. [^{3}H]Ro15-4513 was present at a concentration equal to its K$_d$ value as determined by saturation analysis. For competition assay using rat brain membrane, [^{3}H]Ro15-1788 was used as the radioligand. Data for are from at least three separate experiments performed in duplicate. Results represent mean ± SEM.

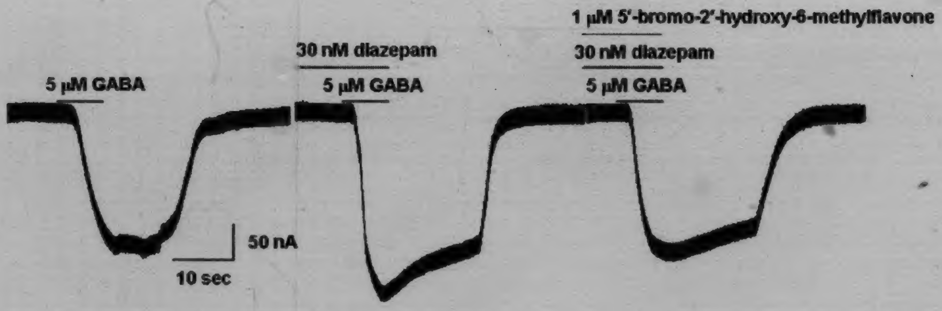

Figure 4-3

Potentiation of the GABA-mediated currents at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors by diazepam is inhibited by a low micromolar concentration of 5′-bromo-2′-hydroxy-6-methylflavone. Current traces recorded from an oocyte expressing recombinant $\alpha_1\beta_2\gamma_2$ wild type $GABA_A$ receptors in response to 5μM GABA. Potentiation of the GABA-mediated current upon perfusion of 30nM diazepam followed by coapplication with 5μM GABA is shown, which is subsequently inhibited by the addition of 1μM 5′-bromo-2′-hydroxy-6-methylflavone.

	Affinity	
α subunit	**Log IC_{50} ± SEM (M)**	**K_i (nM)**
$\alpha_1\beta_2\gamma_2$	-7.92 ± 0.2	12.0
$\alpha_2\beta_2\gamma_2$	-7.20 ± 0.1*	63.1
$\alpha_4\beta_2\gamma_2$	N.D.	N.D.
$\alpha_5\beta_2\gamma_2$	-7.68 ± 0.2	20.9
$\alpha_6\beta_2\gamma_2$	N.D.	N.D.

Table 4-1

Effects of α subunit substitution on the affinity of 5′-bromo-2′-hydroxy-6-methylflavone for $GABA_A$ receptor subtypes. All values are derived from displacement of [^{3}H]Ro15-4513 at a concentration equal to its K_d value for each receptor subtype. N.D. indicates values not determined. Data are from at least three experiments performed in duplicate. Data were analyzed by one-way ANOVA followed by the Dunnett post-test for levels of significance. *P<0.05 compared to wild type $\alpha_1\beta_2\gamma_2$.

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CHAPTER 5

Effects of Amino Acid Substitution at the Histidine 101 Position of the Rat α_1 Subunit on the Binding and Function of Ethyl β-carboline-3-carboxylate (β-CCE), a β-Carboline Benzodiazepine Site Inverse Agonist

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Affinity K_i values for β-CCE tested at $\alpha_1\beta_2\gamma_2$ wild type and mutant $GABA_A$ receptor subtypes were contributed by Dr. J.M.C. Derry.

1. Introduction

The benzodiazepine binding site of the $GABA_A$ receptor is perhaps most widely known with regard to the clinical potency of the benzodiazepine class of drugs (Sieghart, 1995; Korpi et al., 2002). Nevertheless, many other different classes of ligands have also been shown to bind to this important allosteric modulatory site on the $GABA_A$ receptor, including the β-carbolines, the imidazobenzodiazepines, and the cyclopyrrolones (Sigel, 2002). In addition to encompassing a large variety of structural features, these ligands also range the full spectrum of efficacies.

Previous studies examining the benzodiazepine binding site of the $GABA_A$ receptor have outlined the importance of the histidine 101 (rat α_1 subunit) residue to ligand interaction at this site (Wieland et al., 1992; Duncalfe et al., 1996; Davies et al., 1998; Dunn et al., 1999). This loop A residue has been shown to confer differential recognition of classical 1,4-benzodiazepine positive modulators, such as diazepam and flunitrazepam, to $GABA_A$ receptor subtypes based on the specific α-subunit isoform present in the receptor complex (Wieland et al., 1992). As stated earlier, the classification of diazepam-sensitive (DS) includes $GABA_A$ receptor subtypes incorporating the α_1, α_2, α_3, or α_5 isoform, all of which have a histidine residue present at position 101. Conversely, the diazepam-insensitive (DI) classification includes $GABA_A$ receptors incorporating the α_4 or α_6 isoform, both of which have an arginine residue present at this position. Further research has provided evidence that photoaffinity labeling of the $GABA_A$ receptor with [^{3}H]flunitrazepam leads to covalent association of the ligand with the histidine residue at this site in the α subunit, suggesting it is the major site of photoincorporation (Duncalfe et al., 1996).

Previous work in this laboratory has examined the chemical specificity of the residue in this position to determine its effects on benzodiazepine site ligand binding, by using site-directed mutagenesis to create an array of histidine 101 mutants (Davies et al., 1998). Although the amino acid substitutions were found to have differential effects on the binding of various ligands at this site, results suggested that the histidine 101 residue might be more closely involved in recognition properties for agonists and antagonists, as compared to inverse agonists. Indeed, many of the amino acid substitutions examined actually led to an increase in affinity for the imidazobenzodiazepine partial inverse agonist Ro15-4513, whereas decreases in affinity were apparent for the agonists and inverse agonists studied. A subsequent study using this array of histidine 101 mutations investigated the effects of the amino acid substitutions on the functional responses of $GABA_A$ receptors to the benzodiazepine site ligands (Dunn et al., 1999). Changes in both potency and/or efficacy were apparent for all ligands examined although, interestingly, dramatic differences in efficacy were more noticeable for the antagonist Ro15-1788 and the inverse agonist Ro15-4513, as compared to the agonist flunitrazepam (Fig. 5-1). Overall findings concluded that characteristics of the residue present at position 101 are important determinants of both the affinity and efficacy of ligands binding at the benzodiazepine site.

The present study continued investigation of the effects of amino acid substitution at the histidine 101 position on the interaction of ligands at the benzodiazepine binding site. The function of ethyl β-carboline-3-carboxylate (β-CCE), a β-carboline inverse agonist, was examined at recombinant wild type $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, as well as at mutant $GABA_A$ receptors containing a tyrosine, lysine, glutamate, or glutamine substitution at position 101, and findings were compared to previous work from this laboratory which examined the binding affinity of this compound for these receptor subtypes. Results indicate that histidine 101 may be less important to inverse agonist recognition at the benzodiazepine binding site as compared to agonist recognition, and that some of the recognition properties of the benzodiazepine site may be common to ligands sharing the same efficacy, regardless of their structure.

2. Results

2.1 β-CCE binding at wild type and histidine 101 mutant $GABA_A$ receptors

Previous work from this laboratory examined the binding affinity of β-CCE for recombinant wild type and mutant $GABA_A$ receptors (Derry et al., 2004; Derry, unpublished data). Membranes from tsA201 cells were used to carry out competition binding experiments where the wild type rat α_1 subunit, or rat α_1 subunit containing a tyrosine, lysine, glutamate, or glutamine substitution at the histidine 101 site, were transiently expressed in combination with β_2 and γ_2 subunits. [^{3}H]Ro15-4513, which binds to each of these subtypes with high affinity, was used as the radioligand. β-CCE displaced bound radioligand from all of the recombinant preparations with nanomolar affinity. The affinities of β-CCE for recombinant wild type and mutant GABA$_A$ receptor subtypes are summarized in Table 5-1.

2.2 Functional effects of β-CCE at wild type and histidine 101 mutant GABA$_A$ receptors

The functional effects of β-CCE at recombinant GABA$_A$ receptors expressed in *Xenopus laevis* oocytes were examined using two-electrode voltage-clamp electrophysiology. A moderate negative modulation of GABA-evoked currents was observed at the wild type GABA$_A$ receptor; however this could only be observed at concentrations below 10 nM as potentiation attributed to interaction with the loreclezole site masked the effect at higher concentrations (Fig. 5-2A, 5-3A; see Discussion). A negative modulation of GABA-evoked current comparable to that observed at the wild type GABA$_A$ receptor subtype was also seen when β-CCE was applied at GABA$_A$ receptors containing the tyrosine, lysine, or glutamate substitution at position 101 of the α_1 subunit (Fig. 5-2B, 5-3B). In contrast, no effect on the GABA-evoked current was observed when β-CCE was applied at recombinant GABA$_A$ receptors containing the glutamine substitution at position 101 of the α_1 subunit. All electrophysiology data for β-CCE are summarized in Table 5-2.

3. Discussion

The present study examined the effects of mutating the histidine 101 residue in the α_1 subunit on the binding and function of the inverse agonist β-CCE at the GABA$_A$ receptor. Lower affinity was observed for all of the mutations, as compared to the wild type receptor, with the tyrosine and glutamate substitutions having the greatest effects on affinity (decreases of ~20-fold) (Table 5-1). The profile of changes in affinity for β-CCE observed for the four mutations examined is similar to the profiles previously seen with the inverse agonist Ro15-4513 and the antagonist Ro15-1788, where the tyrosine and glutamate substitutions also resulted in lower affinities as compared to the lysine and glutamine substitutions (Davies et al., 1998). It is difficult to speculate as to the reason for the greater loss of affinity with these two substitutions. It is possible that the loss of nitrogen atoms, normally present on the imidazole side moiety of histidine and found in the primary amine side chain group of lysine and the amide side chain group of glutamine, respectively, contributes to the impaired high affinity binding observed with the tyrosine and glutamate substitutions (Fig. 5-4). If this were the case, it is tempting to further speculate that this specific interaction is particular to antagonist and inverse agonist recognition, as a similar profile was not observed for the agonist flunitrazepam (Davies et al., 1998). Nevertheless, at present it cannot be concluded with any certainty that this profile is in fact specific to antagonists and inverse agonists. The possibility that changes in steric or charge-charge interactions, specific to each of these compounds respectively, is responsible for the observed changes in affinity regardless of their efficacy, cannot be excluded. For instance, for the β-carboline antagonist ZK93426, analysis of the profile for the four substitutions examined reveals that, while similar, there is a greater loss of affinity observed with the lysine mutation as compared to the other antagonist and inverse agonists studied (Davies et al., 1998). Further competition studies with antagonists and inverse agonists of different structures would be required to determine with certainty whether binding determinants for benzodiazepine site ligands may in fact be organized in a manner which distinguishes between ligands based on their efficacy.

Functional analysis of β-CCE in the current study revealed that it acts as a negative modulator (inverse agonist) of chloride currents elicited by an EC_{50} concentration of GABA at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Fig. 5-2A, 5-3A). However, addition of higher concentrations of this β-carboline compound (>10 nM) resulted in an apparent reversal of inhibition and potentiation of GABA-evoked current. This observation is in agreement with previous findings which attribute this biphasic action to interaction with the low affinity loreclezole site (Wafford et al., 1994; Wingrove et al., 1994; Stevenson et al., 1995). Subsequent comparisons of potency and efficacy of β-CCE at mutant receptors therefore were made using only observations of the inhibition of GABA response at the wild type receptor seen at lower concentrations (<10 nM). β-CCE negatively modulated GABA-evoked currents at $GABA_A$ receptor subtypes containing the tyrosine, lysine, and glutamate mutations, in a manner comparable to that seen with the wild type receptor (Fig. 5-2B, 5-3B). No significant difference in potency or dramatic change in efficacy was observed. In contrast, no effect on GABA-evoked current was observed when β-CCE was applied at recombinant $GABA_A$ receptors containing the glutamine substitution, suggesting it acts as an antagonist at this mutant receptor subtype (Fig. 5-3B; Table 5-2). This observation parallels previous findings with Ro15-4513, where the inverse agonist also acted as an antagonist at mutant receptors containing the histidine to glutamine substitution (Dunn et al., 1999). However, whereas β-CCE seemed to retain its negative modulator efficacy at the other substitutions examined, Ro15-4513 was shown to act as a partial agonist at these substitutions. This latter observation once again cautions against further speculation that certain binding determinants for the benzodiazepine site might distinguish between ligands based solely on their efficacy.

Despite the continuing advances in understanding the overall structural organization of the benzodiazepine cleft, much remains unknown. How this organization corresponds to functional modulation of $GABA_A$ receptor activity remains to be fully elucidated. Overall, the findings of the current study suggest that changes in efficacy do not parallel changes in apparent affinity for the inverse agonist β-CCE at $GABA_A$ receptor subtypes containing histidine 101 mutations. Nonetheless, certain parallels in findings from this study, with those of previous studies that examined the antagonist Ro15-1788 and the inverse agonist Ro15-4513, allude to possible benzodiazepine site recognition properties based on the efficacy of a ligand. Whether there are similar binding contacts within the benzodiazepine cleft for all of the various classes of benzodiazepine site compounds regardless of their structure or efficacy, or whether the recognition determinants for benzodiazepine site ligands are indeed organized in a manner which can be distinguished according to the specific characteristics of the allosteric modulator in question, is not yet completely understood. It is clear however, particularly with regard to therapeutic potential, that understanding the specificity of drug-receptor interactions at the $GABA_A$ receptor benzodiazepine binding site will greatly enhance the development of pharmacological agents that act at this site with precise and exact effects.

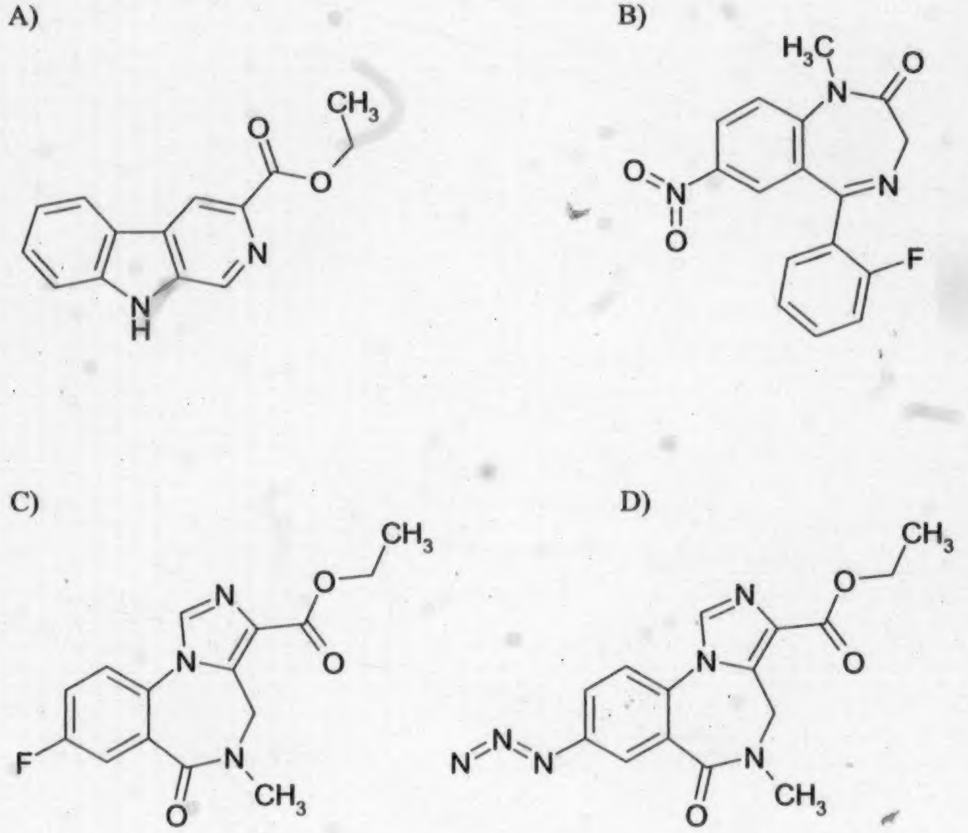

Figure 5-1

Chemical structure of the β-carboline inverse agonist ethyl β-carboline-3-carboxylate (β-CCE) (A). The classical 1,4-benzodiazepine positive modulator flunitrazepam (FNZ) (B), the imidazobenzodiazepine antagonist Ro15-1788 (C) and partial inverse agonist Ro15-4513 (D), are depicted for comparison.

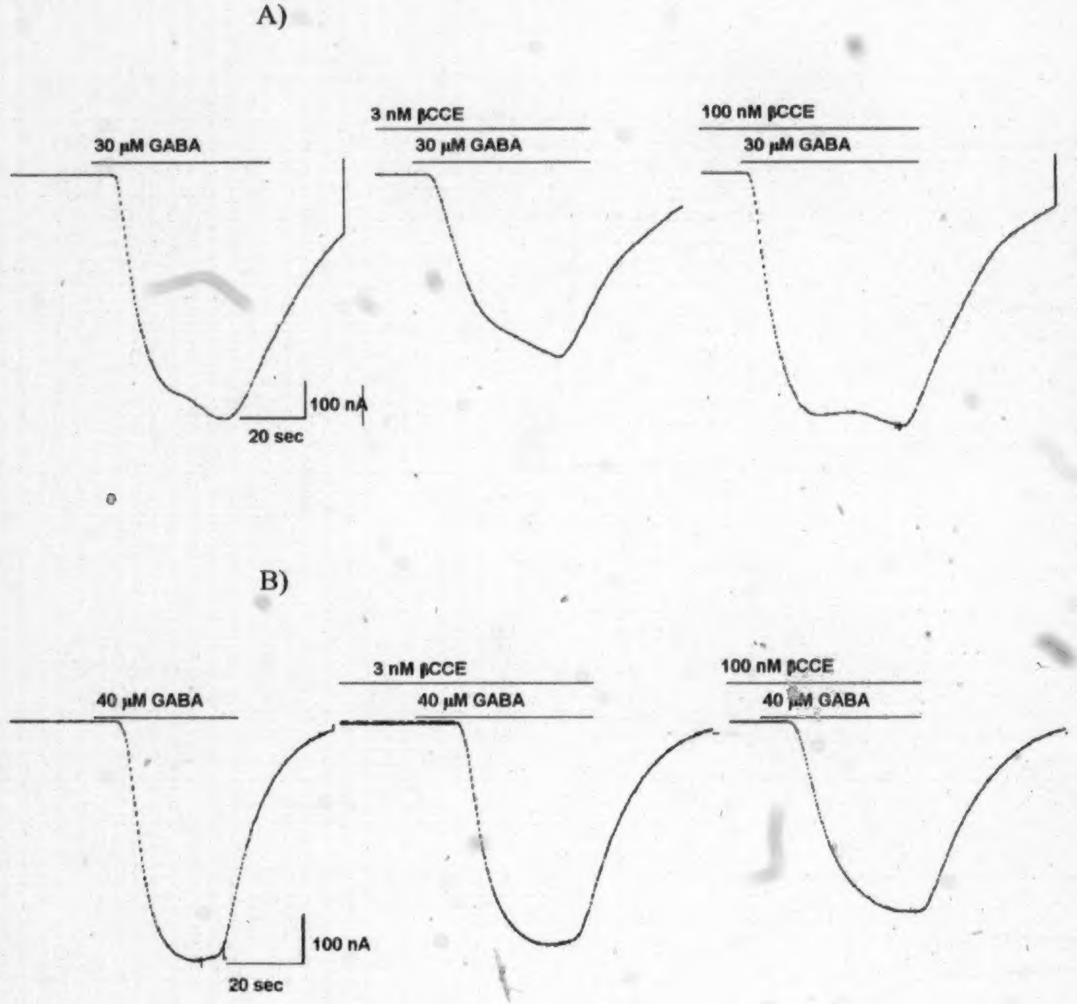

Figure 5-2

Negative modulation of GABA-mediated currents by β-CCE.

(A) Current traces recorded when β-CCE was tested with oocytes expressing recombinant $\alpha_1\beta_2\gamma_2$ wild type $GABA_A$ receptors. A 3 min perfusion of each β-CCE concentration (not shown) was followed by co-application of β-CCE with an EC_{50} concentration of GABA. Note that with addition of higher nanomolar concentrations of β-CCE, a reversal of the inhibition of GABA-mediated current was observed (see Discussion).

(B) Current traces recorded when β-CCE was tested with oocytes expressing recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors containing a tyrosine mutation at position 101 of the α_1 subunit.

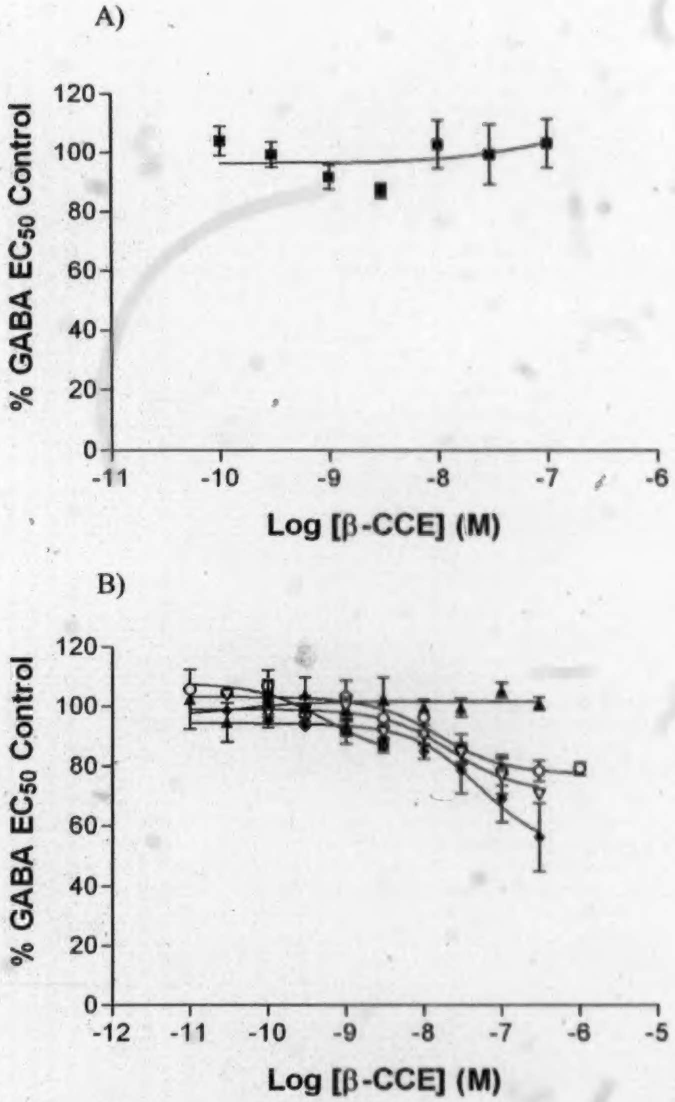

Figure 5-3

(A) The effects of β-CCE on currents induced by an EC_{50} concentration of GABA at the recombinant wild type $\alpha_1\beta_2\gamma_2$ (■) $GABA_A$ receptor subtype are shown. Note the reversal of inhibition with higher nanomolar concentrations of β-CCE (see Discussion).

(B) The effects of β-CCE on currents induced by an EC_{50} concentration of GABA at recombinant $GABA_A$ receptors containing the tyrosine (▽), lysine (◆), glutamate (○), and glutamine (▲) mutations at position 101 of the α_1 subunit are shown. $\alpha_1\beta_2\gamma_2$ (■) is also included for comparison. Data are from observations made from at least three oocytes.

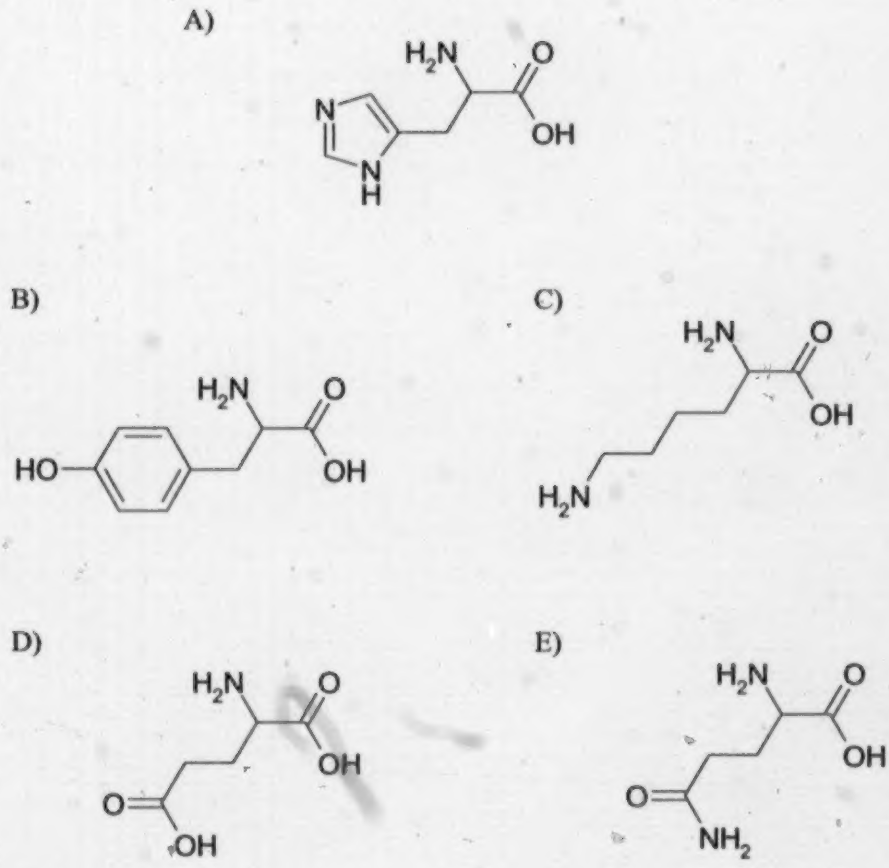

Figure 5-4

Chemical structures of the amino acid mutations examined in this chapter are depicted for comparison of their side chains. The structure of the histidine residue (A), normally present at position 101 of the α_1 subunit, is shown, as well as the structures of (B) tyrosine, (C) lysine, (D) glutamate, and (E) glutamine, which were incorporated as mutations.

	Affinity K_i (nM)
$\alpha_1\beta_2\gamma_2$ wild type[a]	0.4
α_1 subunit mutation[b]	
tyrosine	9.0
lysine	4.4
glutamate	7.2
glutamine	3.3

Table 5-1

Effects of α_1 subunit histidine 101 mutation on the affinity of β-CCE for the $GABA_A$ receptor benzodiazepine site. Affinity K_i values are derived from displacement of [^{3}H]Ro15-4513 at a concentration equal to its K_d value for each receptor subtype.

[a]Derry et al., 2004; [b]Derry, unpublished data.

	Log IC_{50} ± SEM (M)	IC_{50}(95% CI) (nM)
$\alpha_1\beta_2\gamma_2$ wild type	-8.81 ± 0.9	1.55 (0.009-260)
α_1 subunit mutation		
tyrosine	-7.58 ± 0.3	26.3 (5.7-120)
lysine	-7.36 ± 0.1	43.7 (1.4-1400)
glutamate	-7.79 ± 0.2	16.2 (2.1-130)
glutamine	N.D.	N.D.

Table 5-2

Negative modulation of GABA-mediated currents by β-CCE. Data are from observations made from at least three oocytes. Experiments were performed using GABA EC_{50} concentrations. N.D. indicates values not determined.

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CHAPTER 6

Comparison of α_4- versus α_6-Containing GABA$_A$ Receptor Responses to GABA Site Ligands

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Randomly derived chimeras from the α_4 and α_6 subunits were made by Dr. J.M.C. Derry (Derry et al., 2004).

1. Introduction

The $GABA_A$ receptor is considered a key pharmacological candidate with regard to therapeutic interventions targeting neuronal conditions characterized by imbalances of excitation and inhibition. This idea is strengthened by increasing knowledge as to the central role this receptor is postulated to play in mediating fast inhibitory synaptic neurotransmission. There has been a more recent shift in focus, however, with increasing research for drugs targeting $GABA_A$ receptors found at extrasynaptic locations. It is thought that this fraction of $GABA_A$ receptors is responsible for setting the overall inhibitory tone of a brain region by establishing the threshold for the activation of action potentials (Brickley et al., 2001; Mody, 2001). This mechanism of regulating neuronal excitability remains poorly understood, but it is proposed that extrasynaptic receptors respond to spillover of synaptically-released GABA, non-vesicular released GABA, or ambient levels of GABA (Attwell et al., 1993; Scanziani, 2000; Wei et al., 2003; Cavelier et al,. 2005). Pharmacological agents which are able to selectively target extrasynaptic $GABA_A$ receptor subtypes then, may prove useful for the treatment of neuronal disorders characterized by an underlying hyperexcitability, such as epilepsy and insomnia. As mentioned previously, Gaboxadol (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol; THIP) is currently in late-stage investigations as a treatment for insomnia (Wafford and Ebert, 2006). Unlike classical benzodiazepine therapy, which largely targets $GABA_A$ receptors located at the postsynaptic membrane, THIP demonstrates increased selectivity for extrasynaptic $GABA_A$ receptor subtypes (Brown et al., 2002; Stórustovu and Ebert, 2006). It is this functional selectivity which has renewed interest in GABA site agonists as possible therapeutic agents.

Extrasynaptic $GABA_A$ receptors are suggested to contain mainly α_4, α_5, α_6, and δ subunits (Mody, 2001; Whiting, 2003). The incorporation of the α_4 or α_6 subunit in a $GABA_A$ receptor is known to confer insensitivity to classical 1,4-benzodiazepines such as diazepam (DI subtypes) based on the presence of an arginine residue at position 101 (Wieland et al., 1992; see Chapter 1). These two α subunits are considered closely related, sharing the highest homology of all the α subunit isoforms and exhibiting similar functional properties (Ymer et al., 1989; Wisden et al., 1991; Wafford et al., 1996). Nevertheless, the α_4 and α_6 subunits have been shown to differentially affect the pharmacology of $\mathrm{GABA_A}$ receptor subtypes. Receptors have been shown to produce dramatically different responses to the GABA site agonists THIP and piperidine-4-sulfonic acid (P4S) depending on which of these two subunits is present within the pentamer (Wafford et al., 1996). In addition, despite the insensitivity to classical 1,4-benzodiazepines observed at both α4- and α6-containing $\mathrm{GABA_A}$ receptor subtypes, previous work in this laboratory has revealed that these subunits differentially affect the affinity of other ligands for the benzodiazepine site (Derry et al., 2004). Randomly derived chimeras created from the α_4 and α_6 subunits led to the identification of a specific residue that bestows differential affinity for β-carbolines. Taken together, these findings suggest that although these two α subunit isoforms are most homologous to each other, they contain specific domains that contribute to the different pharmacology of the $\mathrm{GABA_A}$ receptors in which they are present.

With increasing interest in the potential for the development of therapeutic agents that target extrasynaptic $\mathrm{GABA_A}$ receptors to affect overall tonic inhibition, further research into the differential effects of GABA site agonists at different receptor subtypes to determine the significance of specific subunits is important. As mentioned above, it has been shown that the GABA site agonists THIP and P4S produce dramatically different responses at the diazepam-insensitive, α_4- versus α_6-containing $\mathrm{GABA_A}$ receptors (Wafford et al., 1996). The present study sought to further characterize the differences in α_4- versus α_6-containing $\mathrm{GABA_A}$ receptor responses using the randomly derived chimeras made previously in this laboratory (Derry et al,. 2004). The agonist responses of the endogenous agonist GABA, and the GABA site agonists THIP and P4S were studied at α_4- and α_6-containing recombinant $\mathrm{GABA_A}$ receptor subtypes expressed in *Xenopus laevis* oocytes using two-electrode voltage-clamp electrophysiology (Fig. 6-1). Chimeric α_6/α_4 recombinant $\mathrm{GABA_A}$ receptors were also studied to determine whether a specific domain could be identified which confers differential sensitivity to the subunits (Fig. 6-2). Results suggest that the overall differential sensitivity of α_4- versus α_6-containing $\mathrm{GABA_A}$ receptors to GABA site ligands may not be as dramatic as previously indicated.

2. Results

2.1 Functional effects of GABA at wild type and chimeric GABA$_A$ receptor subtypes

The functional effects of GABA at recombinant wild type and chimeric GABA$_A$ receptors expressed in *Xenopus laevis* oocytes were compared using two- electrode voltage-clamp electrophysiology (Fig. 6-3, 6-4). Concentration-effect curves to GABA were constructed and revealed a significant five-fold rightward shift in EC$_{50}$ value for the $\alpha_6\beta_2\gamma_2$ subtype (EC$_{50}$= 25.1 μM, 95% CI: 16-39) as compared to the $\alpha_4\beta_2\gamma_2$ subtype (EC$_{50}$= 5.01 μM, 95% CI: 3.5-7.3) (Fig. 6-5A). Hill coefficients were similar for both subtypes. The effects of GABA determined at the chimeric α_6/α_4-containing receptors showed that at the χ141-containing receptor, a significant nine-fold rightward shift in EC$_{50}$ value (EC$_{50}$= 44.9 μM, 95% CI: 32-64) was observed as compared to the wild type $\alpha_4\beta_2\gamma_2$ subtype, whereas no significant difference in potency was determined when compared to the wild type $\alpha_6\beta_2\gamma_2$ subtype. In contrast, no significant difference in the potency of GABA at the χ207-containing receptor (EC$_{50}$= 9.02 μM, 95% CI: 0.96-85) was observed as compared to either the $\alpha_4\beta_2\gamma_2$ or the $\alpha_6\beta_2\gamma_2$ receptor subtypes. Interestingly, the Hill coefficients determined from concentration-effect curves to GABA constructed for both chimeric receptor subtypes were markedly reduced as compared to those observed for the wild type receptor subtypes. Substitution of the β_1 isoform for the β_2 subunit in the wild type α_4- and α_6-containing GABA$_A$ receptor subtypes resulted in no significant difference in GABA potency between the α_4-containing subtypes or between the α_6-containing subtypes (Fig. 6-5B). Interestingly, incorporation of the β_1 isoform did result in a loss of significant difference between the EC$_{50}$ values determined for the α_4- versus the α_6-containing subtypes, as compared to the significant shift observed when the β_2 subunit was present. The functional effects of GABA at recombinant wild type and chimeric GABA$_A$ receptor subtypes are summarized in Table 6-1.

2.2 Functional effects of THIP at wild type and chimeric GABA$_A$ receptor subtypes

Concentration-effect curves constructed for THIP revealed no significant difference in potency between the $\alpha_4\beta_2\gamma_2$ (EC$_{50}$= 154 μM, 95% CI: 39-620) and $\alpha_6\beta_2\gamma_2$ (EC$_{50}$= 286 μM, 95% CI: 250-320) receptor subtypes (Fig. 6-6A). The Hill coefficient observed for the $\alpha_6\beta_2\gamma_2$ receptor subtype was higher than that observed for the $\alpha_4\beta_2\gamma_2$ receptor subtype; however, maximum efficacy of THIP was observed to be greater at the

$\alpha_4\beta_2\gamma_2$ receptor subtype as compared to the $\alpha_6\beta_2\gamma_2$ subtype (Fig. 6-3). The effects of THIP determined at the chimeric α_6/α_4-containing receptors revealed a marked leftward shift in potency of THIP at both the χ141-containing (EC_{50}= 26.5 μM, 95% CI: 12-55) and χ207-containing (EC_{50}= 29.9 μM, 95% CI: 0.43-2100) receptors, which were determined to be significant as compared to the potency of THIP at the wild type $\alpha_6\beta_2\gamma_2$ receptor subtype. In addition, at both the χ141- and χ207-containing receptors, the maximum response to THIP was consistently larger than the maximum response obtained with GABA (Fig. 6-4). Substitution of the β_1 isoform for the β_2 subunit in the wild type α_4- and α_6-containing $GABA_A$ receptor subtypes resulted in no significant difference in THIP potency or efficacy between the α_4-containing subtypes or between the α_6-containing subtypes (Fig. 6-6B). The functional effects of THIP at recombinant wild type and chimeric $GABA_A$ receptor subtypes are summarized in Table 6-1.

2.3 Functional effects of P4S at wild type and chimeric $GABA_A$ receptor subtypes

No significant differences in potency or efficacy were observed for P4S between the $\alpha_4\beta_2\gamma_2$ and $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptor subtypes (Fig. 6-7A). The effects of P4S determined at the chimeric α_6/α_4-containing receptors revealed no significant differences in potency or efficacy for either the χ141- or the χ207-containing receptors as compared to the wild type receptor subtypes. A reduction in Hill coefficient was observed for concentration-effect curves constructed to P4S for both the χ141- and the χ207-containing receptors compared to the wild type receptor subtypes, similar to that observed with GABA. Substitution of the β_1 isoform for the β_2 subunit in the wild type α_4- and α_6-containing $GABA_A$ receptor subtypes resulted in no significant difference in P4S potency or efficacy between the α_4-containing subtypes or between the α_6-containing subtypes (Fig. 6-7B). The functional effects of P4S at recombinant wild type and chimeric $GABA_A$ receptor subtypes are summarized in Table 6-1.

3. Discussion

In the present study, the functional effects of GABA site agonists at α_4- versus α_6-containing $GABA_A$ receptor subtypes were compared. Receptor responses to GABA, THIP and P4S were examined. When comparing the wild type $\alpha_4\beta_2\gamma_2$ and $\alpha_6\beta_2\gamma_2$ receptor subtypes, a difference in potency was only apparent for GABA. Although no change in potency was observed for THIP at these subtypes, it was observed to be a more efficacious ligand at the $\alpha_4\beta_2\gamma_2$ subtype. No changes in potency or efficacy were apparent for P4S between the two subtypes.

Interestingly, examination of the chimeric GABA$_A$ receptor responses to these GABA site agonists did not reveal a clear α_4- versus α_6-like profile. Taking into consideration the previous study using these chimeras (Derry et al., 2004) which clearly revealed α_4- versus α_6-like profiles with regard to differential affinity of ligands for the benzodiazepine site, it was expected that the χ207-containing receptor would produce responses more closely resembling those observed at the wild type $\alpha_6\beta_2\gamma_2$ receptor, as it contains a greater portion of the α_6 subunit than the χ141-containing receptor. Instead, chimeric receptor response profiles were not observed to correlate with either of the wild type receptor subtypes. The potency of GABA at the χ141-containing receptor was shown to be significantly different from that seen at the wild type $\alpha_4\beta_2\gamma_2$ receptor, yet the potency of GABA at the χ207-containing receptor did not differ significantly from either of the wild type receptor subtypes. Noticeably different responses to THIP were apparent for both chimeric receptor subtypes, and their responses to this GABA site agonist seemed to resemble each other more closely than either the wild type $\alpha_4\beta_2\gamma_2$ or $\alpha_6\beta_2\gamma_2$ receptor subtypes. In addition to a marked leftward shift in potency at both chimeric receptors, THIP consistently produced maximum responses that were larger than the maximum response obtained with GABA. It is difficult to speculate as to the basis for the seemingly "gain-of-function" type responses seen with THIP at the chimeric receptor subtypes. It is possible that incorporation of portions of both the α_4 and the α_6 subunits within the chimeric receptor complex leads to structural changes which promote enhanced receptor activation in response to the binding of THIP. For instance, such changes could result in increased frequency of channel opening, increased duration of channel open times, or increased channel conductance levels. Whether these might be due to complex modifications in overall receptor structure, or the alteration of a single amino acid interaction remains unknown. The switch points for both chimeras examined do not lie within loops D, E, or F of the α subunit, which are the regions thought to most strongly interact with the β subunit in forming the agonist binding site. The χ141-containing receptor contains the splice site between loops E and B of the linear sequence, while for the χ207-containing receptor, the splice site is present within the loop C region of the linear sequence (Fig. 6-2). However, recent evidence involving the glycine receptor, where a single-nucleotide polymorphism as a result of RNA-editing leads to the production of a gain-of-function isoform of the receptor that confers high glycine sensitivity, does demonstrate that very small changes in even the primary structure of a receptor can lead to enhanced function (Meier et al., 2005). Additional research outlining the exact contributions of the α_4 and α_6 subunits which could lead to such possible changes in chimeric receptor structure would be necessary to further elucidate the exact mechanism underlying the enhanced responses to THIP. Finally, despite the dramatic difference in chimeric receptor responses to THIP as compared to the wild type receptor subtypes, no differences in potency or efficacy were determined for P4S either between the chimeric subtypes, or as compared to the wild type receptors. Of note, the Hill coefficients for curves constructed for the chimeric receptors to all of the GABA site agonists studied were markedly reduced compared to those constructed for the wild type receptor subtypes, with the exception of THIP at the χ141-containing receptor. This observation is difficult to interpret as it may signify a loss of cooperativity at these receptor subtypes with regard to agonist activation. Interestingly, GABA activation at the χ207-containing receptor subtype does seem to be slower than normally seen at the wild type receptors (Fig. 6-4B).

Taken together, these results suggest the overall differential sensitivity of α_4- versus α_6-containing GABA$_A$ receptors to GABA site ligands may not be as dramatic as previously indicated (Wafford et al., 1996). Incorporation of the β_1 subunit as opposed to the β_2 subunit did not result in significant changes in either the α_4- or α_6-containing receptor response profiles to the GABA agonists studied (Table 6-1), with the exception of a significant decrease in the maximal efficacy of THIP at the $\alpha_4\beta_1\gamma_2$ subtype as compared to the $\alpha_4\beta_2\gamma_2$ subtype. In fact, with respect to GABA, significant differential sensitivity apparent between the α_4- and α_6-containing receptors when the β_2 subunit was present was lost upon incorporation of the β_1 subunit. In contrast to the subtype selectivity based on binding affinity seen with the benzodiazepine site ligands, the affinity of GABA$_A$ receptor agonists may not be chiefly dependent upon subunit composition (Wafford and Ebert, 2006). As stated earlier, the amino acids across the isoforms of the α and β subunits are highly conserved, and as the agonist binding site is located at their interface, lack of selectivity based on this factor would not be surprising. Nevertheless, it cannot be ruled out that the results of the present study, when compared to previous findings (Wafford et al,. 1996), may be due to differences in experimental settings. Possible reasons for the differences in findings include the use of rat cRNA as opposed to human cDNA for injection into *Xenopus laevis* oocytes, the incorporation of the γ_2 long isoform as opposed to the γ_2 short isoform, and potential differences in electrophysiological experimental methodology when examining receptor function.

Functional selectivity based on the extrasynaptic location of $GABA_A$ receptors containing specific subunits does however seem to be a more likely target for pharmacological intervention. As mentioned, extrasynaptic $GABA_A$ receptors are proposed to contain mainly α_4, α_5, α_6, and δ subunits (Mody, 2001; Whiting, 2003). It is suggested that the presence of the δ subunit, which is thought to replace the γ subunit in the pentamer, is associated with localization to extrasynaptic areas (Nusser et al., 1998). It is known that the γ_2 subunit is associated with synaptic anchoring proteins such as gephyrin and GABARAP, involved in the clustering of $GABA_A$ receptors to postsynaptic membranes (Essrich et al., 1998; Wang et al., 1999). It remains to be determined, however, whether the extrasynaptic localization of the δ subunit is due to specific trafficking properties associated with this subunit, or merely a passive mechanism based on an absence of association with anchoring proteins involved in synaptic localization.

The results of the present study were unsuccessful in determining differential sensitivity of α_4- versus α_6-containing $GABA_A$ receptors to GABA site agonists. Nevertheless, in light of recent research findings which demonstrate the effect of extrasynaptic subunit composition on agonist potency and efficacy, there remains the possibility that further research comparing α_4- versus α_6-containing $GABA_A$ receptor responses to GABA site agonists, where the δ subunit is incorporated as opposed to γ_2, may yet reveal differential sensitivity to these ligands. GABA has been shown to be approximately ten-fold more potent at α_6/δ subunit-containing $GABA_A$ receptors as compared to $\alpha_6/\gamma 2$ subunit-containing $GABA_A$ receptors (Santhakumar et al., 2006). Interestingly, it has also been demonstrated that THIP acts with higher potency and efficacy at δ-containing $GABA_A$ receptors, as compared to other receptor subtypes

(Brown et al., 2002; Wafford and Ebert, 2006). More recently, GABA, THIP, and P4S, along with a number of other agonists, have been shown to act with differential potency and/or efficacy at $\alpha_4\beta_3\delta$ versus $\alpha_6\beta_3\delta$ $GABA_A$ receptors (Stórustovu and Ebert, 2006). In this laboratory, recent preliminary investigations of desensitization rates using chimeric δ/γ_2 subunits in combination with α_4 and β_3 subunits has resulted in findings where a clear δ- versus γ_2-like profile was difficult to conclude (You, unpublished data). In comparison, a similar study which also examined desensitization profiles of $GABA_A$ receptors containing chimeric δ/γ_2 subunits, but that incorporated the α_1 subunit as opposed to α_4, led to more conclusive findings as to the subunit domains involved (Bianchi et al., 2001). Taken together, these findings promote further research, particularly with the chimeric α_6/α_4 recombinant $GABA_A$ receptors where the δ subunit has been incorporated, to determine whether subsequent investigations involving different receptor subunit composition may yet identify a specific domain that confers differential sensitivity of α_4- versus α_6-containing $GABA_A$ receptors to GABA site ligands. Continuing research into the selective targeting of extrasynaptic $GABA_A$ receptor subtypes remains an important priority, as the development of GABA site agonists or partial agonists with similar properties to Gaboxadol could provide a unique mechanism for targeting overall tonic inhibition of the brain and, accordingly, various disorders affected by underlying imbalances in excitation and inhibition.

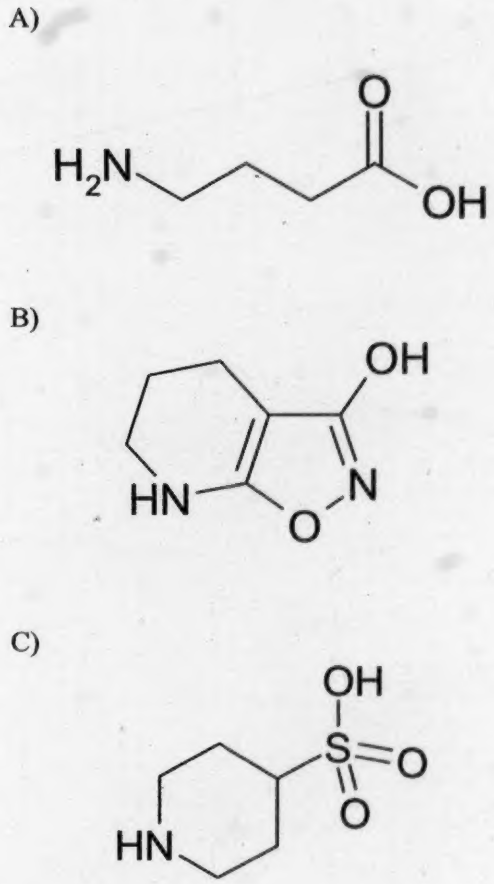

Figure 6-1

Chemical structures of GABA site ligands examined in this chapter. The structures of (A) γ-aminobutyric acid (GABA), (B) 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), and (C) piperidine-4-sulfonic acid (P4S) are depicted for comparison.

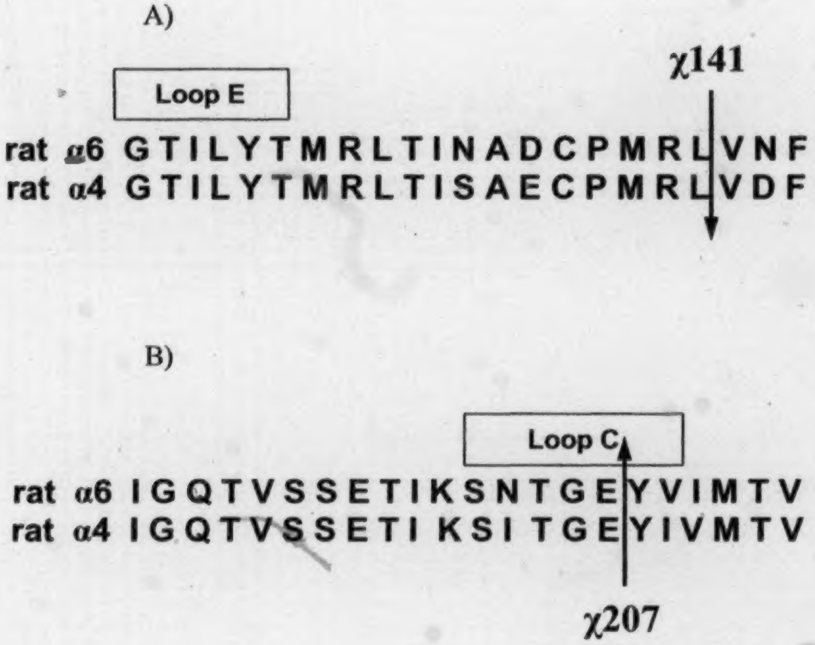
A)
χ141
Loop E
rat α6 GTILYTMRLTINADCPMRLVNF
rat α4 GTILYTMRLTISAECPMRLVDF
B)
Loop C
rat α6 IGQTVSSETIKSNTGEYVIMTV
rat α4 IGQTVSSETIKSITGEYIVMTV
χ207

Figure 6-2

Sequence alignment of the α_6 and α_4 GABA$_A$ receptor subunit regions identifying the switch points for the α_6/α_4 chimeric subunits. Numbering of the switch points represents the last residue of the α_6 sequence prior to the switch point.

(A) The χ141 chimeric subunit switch point is located downstream of the amino acid segment encoding loop E.

(B) The χ207 chimeric subunit switch point is located within the amino acid segment encoding loop C.

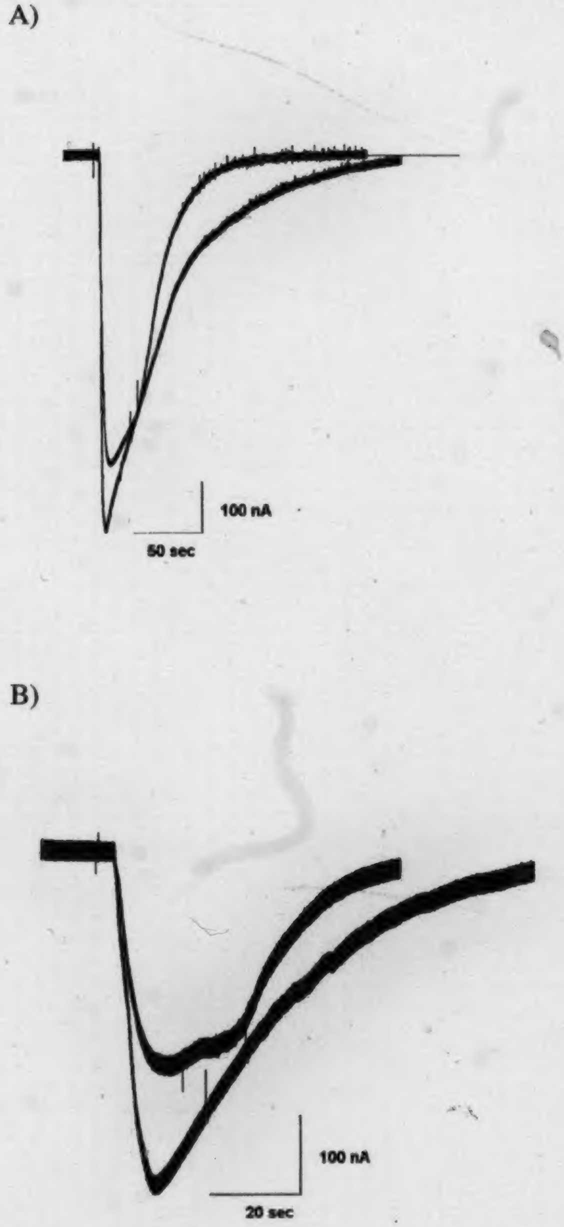

Figure 6-3

Current traces comparing 3mM concentrations of GABA (*black*) and THIP (*red*) tested at oocytes expressing recombinant wild type (A) $\alpha_4\beta_2\gamma_2$ and (B) $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptors.

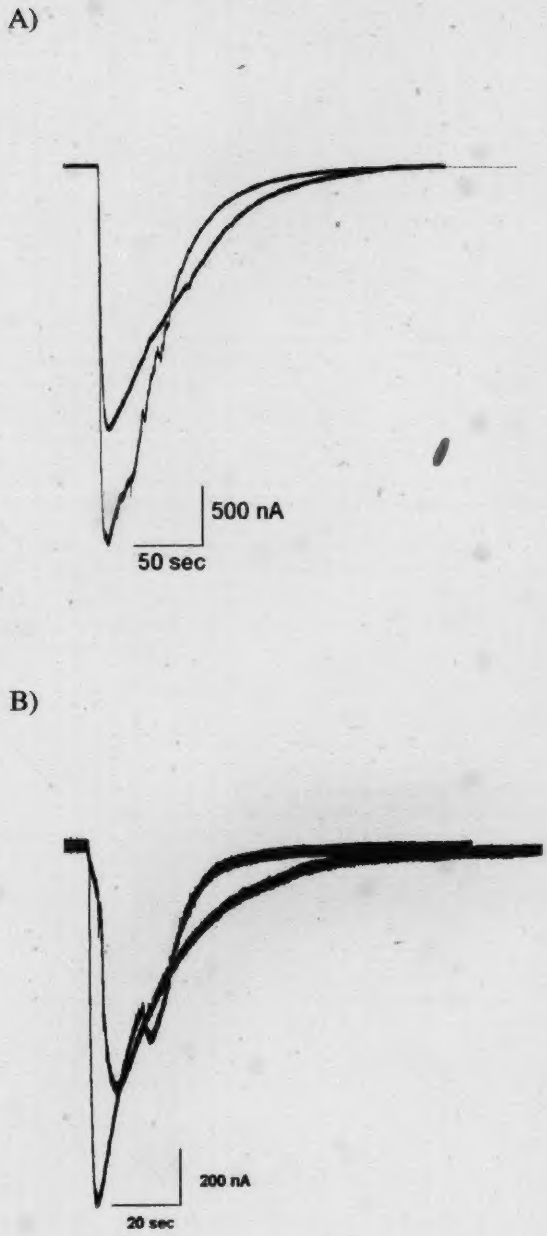

Figure 6-4

Current traces comparing 3mM concentrations of GABA (*black*) and THIP (*red*) tested at oocytes expressing recombinant (A) χ141- and (B) χ207-containing $GABA_A$ receptors.

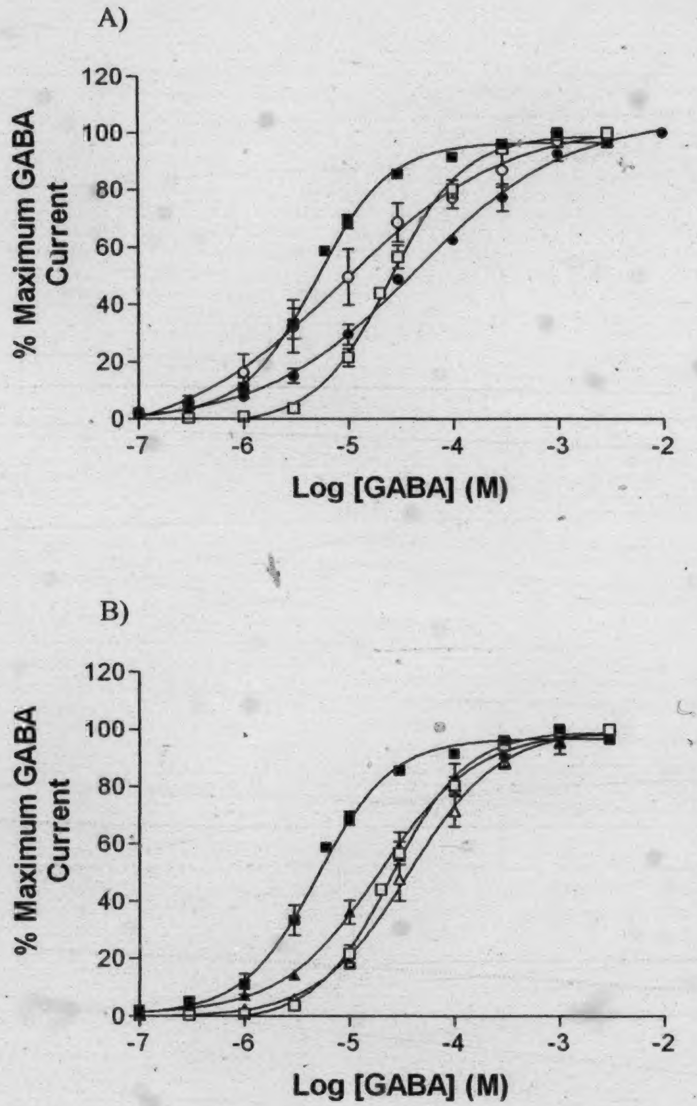

Figure 6-5

Concentration-effect curves for GABA_A receptor activation by GABA. The receptor subtypes examined include recombinant wild type $\alpha_4\beta_2\gamma_2$ (■) and $\alpha_6\beta_2\gamma_2$ (□), which are graphed with (A) $\alpha_{6\text{-}4}(\chi141)\beta_2\gamma_2$ (●), $\alpha_{6\text{-}4}(\chi207)\beta_2\gamma_2$ (○), and (B) wild type $\alpha_4\beta_1\gamma_2$ (▲), wild type $\alpha_6\beta_1\gamma_2$ (Δ), respectively, for comparison. Data for each receptor subtype are from observations made from at least three oocytes. Results represent mean ± SEM.

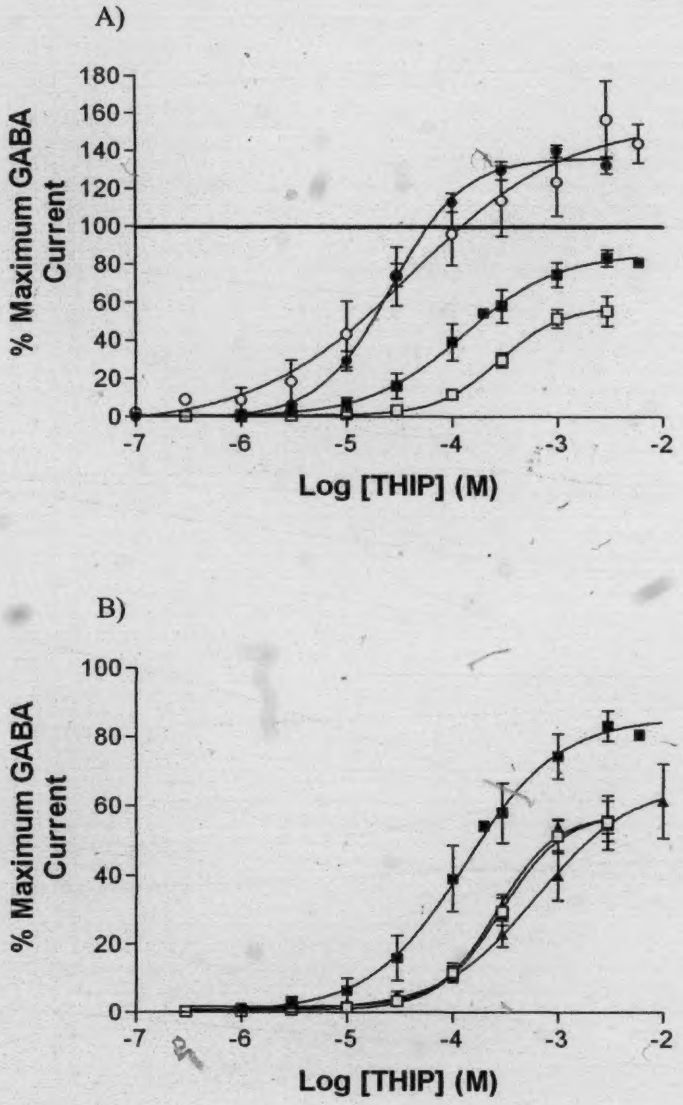

Figure 6-6

Concentration-effect curves for $GABA_A$ receptor activation by THIP. The receptor subtypes examined include recombinant wild type $\alpha_4\beta_2\gamma_2$ (■) and $\alpha_6\beta_2\gamma_2$ (□), which are graphed with (A) $\alpha_{6-4}(\chi141)\beta_2\gamma_2$ (●), $\alpha_{6-4}(\chi207)\beta_2\gamma_2$ (○), and (B) wild type $\alpha_4\beta_1\gamma_2$ (▲), wild type $\alpha_6\beta_1\gamma_2$ (Δ), respectively, for comparison. Data for each receptor subtype are from observations made from at least three oocytes. Results represent mean ± SEM.

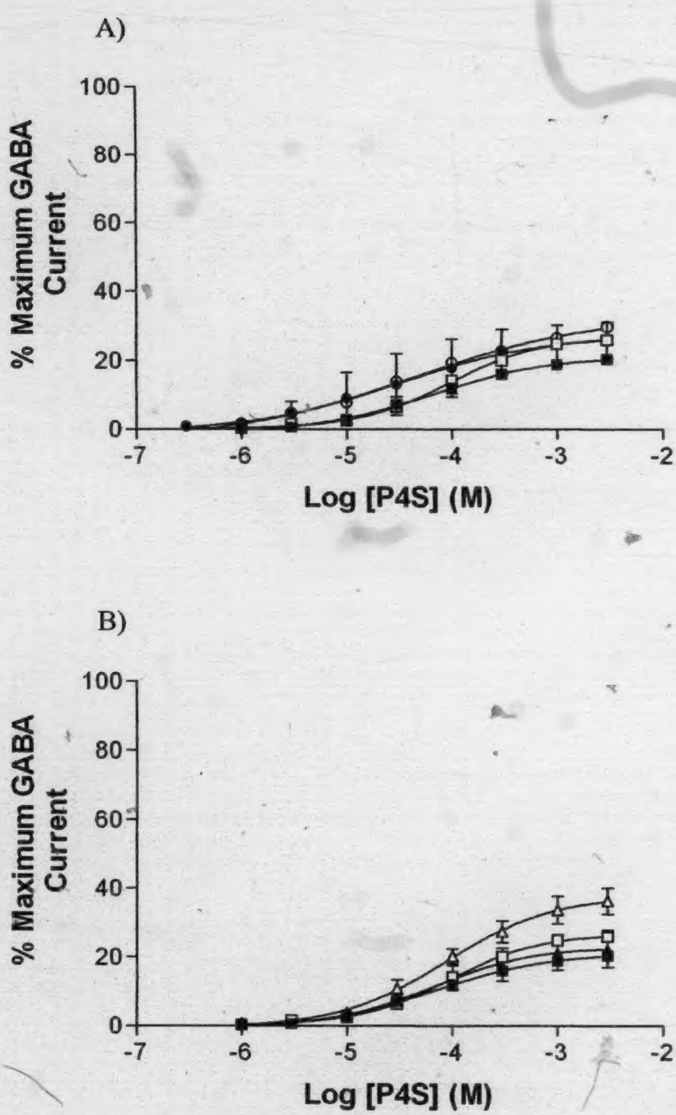

Figure 6-7

Concentration-effect curves for $GABA_A$ receptor activation by P4S. The receptor subtypes examined include recombinant wild type $\alpha_4\beta_2\gamma_2$ (■) and $\alpha_6\beta_2\gamma_2$ (□), which are graphed with (A) $\alpha_{6\text{-}4}(\chi 141)\beta_2\gamma_2$ (●), $\alpha_{6\text{-}4}(\chi 207)\beta_2\gamma_2$ (○), and (B) wild type $\alpha_4\beta_1\gamma_2$ (▲), wild type $\alpha_6\beta_1\gamma_2$ (Δ), respectively, for comparison. Data for each receptor subtype are from observations made from at least two oocytes. Results represent mean ± SEM.

	EC_{50} (µM)	95% C.I.	Max. Efficacy (%)	n_H
GABA				
$\alpha_4\beta_2\gamma_2$	5.01	(3.5-7.3)	100	1.2 ± 0.1
$\alpha_6\beta_2\gamma_2$	25.1^{*}	(16-39)	100	1.2 ± 0.1
$\alpha_{6\text{-}4}(\chi141)\beta_2\gamma_2$	44.9^{*}	(32-64)	100	0.6±0.05
$\alpha_{6\text{-}4}(\chi207)\beta_2\gamma_2$	9.02	(0.96-85)	100	0.5 ± 0.1
$\alpha_4\beta_1\gamma_2$	20.1	(8.1-50)	100	0.9 ± 0.1
$\alpha_6\beta_1\gamma_2$	37.0^{*}	(14-97)	100	1.0 ± 0.1
THIP				
$\alpha_4\beta_2\gamma_2$	154	(39-620)	86 ± 7	1.0 ± 0.3
$\alpha_6\beta_2\gamma_2$	286	(250-320)	58 ± 4	1.5 ± 0.3
$\alpha_{6\text{-}4}(\chi141)\beta_2\gamma_2$	26.5^{**}	(13-55)	136 ± 2	1.3 ± 0.1
$\alpha_{6\text{-}4}(\chi207)\beta_2\gamma_2$	29.9^{**}	(0.43-2100)	153 ± 17	0.6 ± 0.2
$\alpha_4\beta_1\gamma_2$	698	(61-8000)	66 ± 8	1.0 ± 0.3
$\alpha_6\beta_1\gamma_2$	269	(110-650)	57 ± 2	1.6 ± 0.2
P4S				
$\alpha_4\beta_2\gamma_2$	64.3	$(0.021\text{-}2.0*10^{5})$	21 ± 2	0.8 ± 0.2
$\alpha_6\beta_2\gamma_2$	95.7	(67-140)	27 ± 1	1.0 ± 0.1
$\alpha_{6\text{-}4}(\chi141)\beta_2\gamma_2$	31.9	$(1.3*10^{-12}\text{-}1300)$	27 ± 8	0.6 ± 0.6
$\alpha_{6\text{-}4}(\chi207)\beta_2\gamma_2$	42.5	N.D.[a]	33	0.5
$\alpha_4\beta_1\gamma_2$	68.4	(20-230)	23 ± 3	0.9 ± 0.5
$\alpha_6\beta_1\gamma_2$	87.9	(68-110)	38 ± 3	0.8 ± 0.2

Table 6-1

Concentration-effect relationships for $GABA_A$ receptor activation by GABA, THIP, and P4S on receptor subtypes expressed in *Xenopus* oocytes. The currents evoked by a range of GABA, THIP, and P4S concentrations, respectively, were tested to determine the concentrations for half-maximal activation (EC_{50}), maximum efficacy, and Hill slope (n_H). Data are from observations made from at least three oocytes, except for P4S where data are from observations from two oocytes. Data were analyzed by one-way ANOVA followed by the Dunnett post-test for levels of significance. $^{*}P<0.05$ compared to wild type $\alpha_4\beta_2\gamma_2$. $^{**}P<0.05$ compared to wild type $\alpha_6\beta_2\gamma_2$.

[a] Data for P4S at the $\alpha_{6\text{-}4}(\chi207)\beta_2\gamma_2$ receptor subtype was preliminary and therefore confidence intervals and standard error were not determined.

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CHAPTER 7

General Discussion

The $GABA_A$ receptor remains a prime target for pharmacological intervention. Expanding knowledge with regard to the central role this receptor plays in mediating fast synaptic inhibitory transmission, and more recent findings as to its role in setting the overall inhibitory tone of brain regions via extrasynaptic locations, has certainly increased its potential as a pharmacological target. This receptor continues to be investigated with respect to treatments targeting neuronal disorders, particularly those associated with imbalances of excitation and inhibition. However, the search for novel and enhanced therapeutic agents has become increasingly dependent upon an improved understanding of the specificity of drug-receptor interaction. For instance, as discussed previously, the classical 1,4-benzodiazepines, while widely prescribed for the treatment of disorders such as epilepsy, anxiety, and insomnia, are often associated with unwanted side effects as well as the development of tolerance, dependence, and withdrawal symptoms (Korpi et al., 2002; see Chapter 1). The development of agents which act with specificity at a defined receptor target, thus minimizing or even eliminating unwanted side effects that result from other nonspecific interactions, has therefore become a primary objective. It is recognized then, that an improved understanding of the structure-function relationship of the $GABA_A$ receptor, and how this relates to drug interaction at specific sites on the receptor, will be crucial to achieving success in the design of such pharmacological agents.

The discovery of the AChBP and the development of Changeux's loop model made a significant contribution to the concept of the structural organization of the $GABA_A$ receptor (see Chapter 1). This is particularly true with regard to the present understanding of the structure of the benzodiazepine binding site. Best described as a cleft or pocket within which ligand binding takes place, it is now widely believed that ligand binding domains at subunit interfaces include discrete regions or "loops" of amino acids. In the case of the $GABA_A$ receptor in particular, this includes loops A, B, and C of the α subunit which interface with loops D, E, and F of the γ subunit (Galzi and Changeux, 1994; Changeux and Edelstein, 1998; Corringer et al., 2000; Cromer et al., 2002). More specifically, the identity of specific amino acid residues located at the cavity formed by the α-γ interface, which may be important to the binding of benzodiazepine site ligands, continue to be clarified (Grutter and Changeux, 2001; Cromer et al., 2002; Trudell, 2002).

A review of findings from this laboratory, including those of the preceding chapters, shows the importance of the contribution of the histidine 101 residue (loop A, rat α_1 subunit), and to a lesser extent the serine 205 residue (loop C, rat α_1 subunit), to the binding of various benzodiazepine site ligands of differing structures and efficacies (Fig. 7-1, 7-2, 7-3). As stated previously, many different classes of ligands bind at the benzodiazepine binding site, together encompassing a large variety of structural features and ranging the full spectrum of efficacies (Sigel, 2002). This factor has raised questions as to how the structural organization of the benzodiazepine binding cleft corresponds to the functional modulation of the receptor observed upon interaction of a ligand with this site. For instance, are certain binding contacts within the benzodiazepine cleft shared by certain ligands in a manner which can be linked to specific properties shared by those ligands? Specifically, could specific sub sites for certain ligands be present within the benzodiazepine cleft based on recognition characteristics? If this is indeed the case, can specific amino acid residues be identified as binding contacts which are associated with the particular efficacy and/or structure of a benzodiazepine site ligand?

To further investigate these questions, a review of radioligand binding studies from this laboratory which involved site-directed mutagenesis of the histidine 101 residue or the serine 205 residue was performed. As discussed in earlier chapters, histidine 101 has been shown to confer sensitivity to classical benzodiazepine agonists, providing the basis for the DS versus DI classification system (Wieland et al., 1992). This laboratory has since investigated the binding of various benzodiazepine site ligands at an array of histidine 101 mutant receptors (Davies et al., 1998; Dunn et al., 1999; Davies et al., 2000; Derry, unpublished data; Hansen et al., 2005; Hansen, unpublished data). In the present review, these findings are compared with radioligand binding studies investigating the binding of various benzodiazepine site ligands at $GABA_A$ receptors containing a mutation at the serine 205 residue (Derry et al., 2004). Changes in affinity for the various benzodiazepine site ligands at $GABA_A$ receptors containing the histidine 101 or serine 205 mutations, as compared to wild type $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, were analyzed (Table 7-1). Together, these data were examined to determine whether the amino acids investigated play a key role in the recognition of certain benzodiazepine site ligands based on their efficacy and/or structure.

For the histidine 101 mutant receptor subtypes examined, an overall loss of affinity for benzodiazepine site ligands was seen at receptors containing the tyrosine, lysine, and glutamate substitutions. While this finding was observed for positive modulator, antagonist, and negative modulator benzodiazepine site ligands, the loss of affinity was shown to be greater for ligands characterized as positive modulators (Fig. 7-1). In addition, the only exception to this general observation was for the imidazobenzodiazepine negative modulator Ro15-4513, where affinity was shown to actually increase at $GABA_A$ receptors containing the lysine and glutamate substitutions, as compared to the wild type $GABA_A$ receptor. Interestingly, despite these increases in affinity, the profile of affinity changes for Ro15-4513 still resembled that seen with the other negative modulator examined, the β-carboline β-CCE, where the tyrosine and glutamate substitutions also resulted in lower affinities as compared to the lysine (and glutamine) substitution (see Chapter 5).

In general, a loss of affinity for benzodiazepine site ligands (with the exception of Ro15-4513) was also observed at receptors containing the glutamine substitution. However, for the antagonists and negative modulators examined, the shifts in affinity were much smaller as compared to those seen at receptors containing the tyrosine, lysine, and glutamate substitutions. Comparatively, the shifts in affinity were generally also smaller for positive modulators at receptors containing the glutamine substitution as opposed to those observed with the tyrosine, lysine, and glutamate substitutions. In contrast however, the loss of affinity observed with the glutamine mutation was much higher for positive modulator benzodiazepine site ligands, as compared to antagonist and negative modulator ligands, suggesting changes associated with this mutation have a greater effect on the interaction of positive modulators with the benzodiazepine binding site. Finally, for diazepam and zopiclone (positive modulators), and 5′-bromo-2′-hydroxy-6-methylflavone and amentoflavone (antagonists), a complete loss of affinity was observed at $GABA_A$ receptors containing the alanine mutation. This observation demonstrates that the substitution of histidine 101 with an alanine residue particularly affects the interaction of benzodiazepine positive modulators and antagonists with the benzodiazepine site. It is possible that this mutation might particularly affect the binding of classical benzodiazepine agonists and flavonoids at the benzodiazepine site, due to changes in the electrochemical character of this region. Conversely, it is possible that the alanine mutation results in dramatic changes to the overall structure of the benzodiazepine site, as the methyl group side chain of alanine is small in size compared to the imidazole side moiety of histidine, which subsequently affects the ability of benzodiazepine site ligands in general to bind with high affinity (see Chapter 4). Further radioligand binding studies with benzodiazepine site ligands of different structures and efficacies at $GABA_A$ receptors containing this particular mutation would allow us to better discriminate between the aforementioned explanations. Comparatively, the use of receptor modeling techniques to analyze the effects of the histidine to alanine substitution at this position might allow for a prediction as to whether this mutation could disrupt the overall structure of the binding site, or whether the specific binding of ligands of a particular structure or efficacy would more likely be affected by the change. Results demonstrating the affinity of the various benzodiazepine site ligands examined for recombinant wild type and mutant $GABA_A$ receptor subtypes containing the histidine 101 substitutions are summarized in Table 7-1.

Review of the affinity of benzodiazepine site ligands at $GABA_A$ receptors containing an asparagine mutation at the serine 205 position, as compared to wild type $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, revealed a greater loss of affinity for the imidazobenzodiazepine compounds Ro15-1788 and Ro15-4513 (~19-fold and ~38-fold respectively), than for the classical benzodiazepine flunitrazepam and the β-carboline β-CCE. This finding suggests that imidazobenzodiazepine recognition, in particular at the benzodiazepine binding site, is more specifically affected by changes to this residue (Derry et al., 2004). Interestingly, the asparagine substitution led to a slight increase in affinity for flunitrazepam, suggesting that, in contrast to the antagonist and negative modulators examined, mutation at this residue does not significantly affect classical benzodiazepine agonist interaction with the benzodiazepine site (Table 7-1).

Taken together, the data incorporated from these previous radioligand binding studies demonstrate that while it cannot be concluded that histidine 101 is not important to antagonist and negative modulator interaction at the benzodiazepine site, the role this loop A residue plays in positive modulator interaction at this site is likely of greater significance. Despite differences in ligand structure, mutation of this residue had similar effects on recognition of the β-carboline ZK93423 and the cyclopyrrolone zopiclone, in addition to the classical benzodiazepine agonists flunitrazepam and diazepam, suggesting that the role of histidine 101 may be predominantly linked to the efficacy of the benzodiazepine site ligand being bound. In contrast, while mutation of the serine 205 did affect antagonist and negative modulator recognition as compared to the positive modulator flunitrazepam, the considerable loss of affinity observed for the imidazobenzodiazepine compounds in particular suggest that this loop C residue probably plays an important role in the benzodiazepine site binding of ligands sharing similar structural properties. Further radioligand binding studies with $GABA_A$ receptors containing the serine 205 mutation which examine a broader range of compounds varying in structure and efficacy are needed to more definitively elucidate the exact nature of the interaction of this residue with benzodiazepine site ligands.

While review of the above findings does not conclusively establish the idea of sub sites for specific ligands within the benzodiazepine binding cleft, it does provide evidence that specific amino acid residues may in fact act as binding contacts that are associated particularly with the efficacy or structure of a benzodiazepine site ligand. Continued research into the precise mechanism by which this loop A and loop C residue interact with different compounds might further differentiate their respective contributions to ligand recognition at this site. Nevertheless, it must be remembered that the benzodiazepine cleft is comprised structurally of domain contributions from both the α and γ subunits of the $GABA_A$ receptor, and that determinants from both of these subunits at the interface are likely involved in a complex mechanism that underlies recognition of various ligands. It is hoped, however, that affinity studies incorporating site-directed mutagenesis of key residues at the benzodiazepine cleft, such as those reviewed above, will allow for small elucidations of residue contributions that will combine to ultimately further overall understanding of benzodiazepine site ligand recognition.

The elucidation of single residue contributions to ligand recognition may be particularly useful in the context of receptor modeling studies. A number of amino acid residues thought to be important for ligand interaction at the benzodiazepine site have been identified on both the α and γ subunits of the $GABA_A$ receptor, based on the consequences of point mutations (Sigel, 2002). There are limitations to the conclusions which can be drawn from the results of affinity studies (such as those reviewed above) and electrophysiological studies which incorporate site-directed mutagenesis to analyze the effect of specific point mutations on ligand binding and function at the benzodiazepine site. While dramatic changes in the binding affinity of ligands and/or the effects of these ligands on overall receptor function may be observed as a result of a particular mutation, it can only be speculated as to whether these changes are due to a direct mechanism of interaction between the ligand and the amino acid residue being studied, or whether an indirect allosteric effect is responsible. Yet the identification of key residues by point mutation studies can be further investigated by more recent approaches in studying the benzodiazepine site, such as the substituted-cysteine accessibility method (SCAM). This technique can provide evidence of a specific residue being more accessible to a covalent reaction involving cysteine modification, and therefore the likelihood of that residue being present in or near the benzodiazepine binding pocket. In addition, benzodiazepine site ligands have been used in conjunction with this method to determine whether a competitive interaction between the ligand and the modifying agent occurs at a particular residue, which in turn would indicate that the residue is likely part of a subdomain within the pocket for that particular benzodiazepine site ligand (Teissére and Czajkowski, 2001). The secondary structure of segments of amino acids can also be confirmed from the results of such experiments, and subsequently further incorporated into receptor models. Thus affinity studies and electrophysiological studies involving point mutations can act as important precursor studies, whereby key residues at the benzodiazepine site are identified that subsequently, using more advanced techniques as they become available, can lead to an elucidation of not only the potential contributions of a residue at the site, but also a prediction of the overall structure of a particular benzodiazepine binding site domain. The use of computer-generated structural models of the $GABA_A$ receptor provides a powerful tool by which the findings from experimental studies that have incorporated mutations can be combined and tested to gain further insight into the structure-function relationship of the receptor as a whole (Ernst et al., 2003). While it is important to be aware of the limitations of computer models with regard to potential inaccuracies and uncertainties, this technique nonetheless offers a method for testing structural ideas developed from biochemical data, and in turn provides a mechanism for designing subsequent experimental studies that can investigate rational mutations based on generated hypotheses.

Ultimately, the comprehensive understanding of the total structure of the GABA$_A$ receptor will continue to be gained from the findings and predictions of a variety of experimental approaches, including those discussed above, which contribute to receptor models. For instance, with respect to models of flavonoid binding at the benzodiazepine binding site, ongoing development of quantitative structure-activity relationship (QSAR) models has focused on the fitting of flavonoids possessing different substituents within these models in the attempt to explain flavonoid efficacy as a function of the interactions between the ligand and the receptor at the molecular level (Dekermendjian et al., 1999; Marder et al., 2001; Kahnberg et al., 2002; Hong and Hopfinger, 2003). These have incorporated previous experimental findings regarding important substituent positions on the flavonoid backbone, including the 6,3′-disubstitution and the 2′-hydroxyl substitution (see Chapter 4). While questions remain as to the use of diazepam as opposed to other benzodiazepine site ligands for the superimposition of flavonoids in these models, findings from affinity and electrophysiological studies with flavonoids, such as those in the preceding chapters (see Chapter 3 and 4; Hansen et al., 2005), may help to clarify how closely flavonoid interaction at the benzodiazepine site mirrors that of diazepam and/or other ligands and thereby refine future models. Indeed, comparative modeling studies of the benzodiazepine site, and the GABA$_A$ receptor in general, continue to be facilitated by findings from many types of experiments, including those from photoaffinity labeling studies, from affinity and functional studies that examine subtype-specific differences as well as the consequences of single-point mutations using recombinant techniques, from the discovery and determination of the crystal structure of AChBP and finally, from studies employing newer techniques such as SCAM. It is the combined incorporation of data acquired through many varied approaches in future receptor modeling studies that will likely lead to the most extensive understanding of $GABA_A$ receptor structure and function.

Conclusions

Studies such as those described in the preceding chapters illustrate the use of recombinant expression and site-directed mutagenesis to investigate how subtype diversity and point mutations affect the affinity and function of $GABA_A$ receptor ligands at $GABA_A$ receptors. Heterologous expression of defined receptor subtypes in a system such as the *Xenopus* oocyte provides a means by which inherent properties of subunit isoforms can be associated with the pharmacological properties of a ligand at a particular $GABA_A$ receptor subtype (Kapur et al., 2006 in press). In addition, the use of chimeric subunits (Moore and Blakely, 1994; Boileau et al., 1998; Derry et al., 2004) or site-directed mutagenesis (Davies et al., 1998) provides powerful techniques by which pharmacological properties of a ligand at the $GABA_A$ receptor can be linked to specific domains of a subunit or the characteristics of a single amino acid respectively. The use of these techniques in collaboration with affinity and functional studies (such as the radioligand binding experiments and the two-electrode voltage clamp experiments described in the previous chapters) have permitted continuing clarification of the structure-function relationship of the $GABA_A$ receptor.

With regard to $GABA_A$ receptor ligands, these types of investigations produce not only a better understanding of pharmacological interaction at the receptor, but also contribute knowledge necessary for the design and synthesis of agents which act with greater specificity and/or efficacy. For instance the design and synthesis of high affinity flavonoid derivatives, such as 5′-bromo-2′-hydroxy-6-methylflavone, which bind to the benzodiazepine site with higher affinity than naturally occurring flavonoids like amentoflavone, have furthered understanding of flavonoid interaction in general at the benzodiazepine site and advanced modeling studies attempting to definitively confirm these interactions (see Chapters 3 and 4; Kahnberg et al., 2002). One challenge facing these efforts is the observation that changes in affinity do not always appear to parallel changes in efficacy. Thus while studies with 5′-bromo-2′-hydroxy-6-methylflavone have helped to clarify flavonoid binding at the benzodiazepine binding site of the $GABA_A$

receptor, the lack of intrinsic efficacy of this compound at the receptor does not wholly permit significant elucidations as to non-benzodiazepine site-mediated interactions seen with naturally occurring flavonoids. This limitation is also evident in studies comparing the effects of histidine 101 mutation on negative modulator activity at the benzodiazepine site. Whereas affinity changes for the mutations examined were shown to follow a similar profile for the imidazobenzodiazepine Ro15-4513 and the β-carboline β-CCE, Ro15-4513 displayed partial agonist efficacy at some mutations while β-CCE seemed to retain its negative modulator efficacy (see Chapter 5; Dunn et al., 1999).

This observation encourages caution in speculating how changes in $GABA_A$ receptor structure or $GABA_A$ receptor ligand structure that have been shown to affect affinity will affect overall functional modulation of the $GABA_A$ receptor. Nonetheless, there remains a vast potential for findings from these types of studies to further the comprehension of the structure-activity relationship of the $GABA_A$ receptor. It is hoped that this knowledge will contribute to increased understanding of the physiological role of the $GABA_A$ receptor in the central nervous system, and to the design and synthesis of novel pharmacological agents that act at this receptor to target disorders with underlying imbalances of excitation and inhibition, which improve upon the precision and efficacy of existing therapeutics.

Limitations and Future Directions

Limitations with studies presented in the preceding chapters included methodological challenges. Although transient transfection of tsA 201 cells normally resulted in excellent receptor expression as determined through radioligand binding checks, there were periods throughout the course of these studies where atypical factors led to occasions of poor cell growth and/or poor receptor expression. In most circumstances, these issues were resolved and resulted only in minor setbacks to the gathering of results; however in certain instances, irresolution of the problem over an extended period led to the inability to fully complete particular investigations. This was especially the case for the examination of the interaction of 5′-bromo-2′-hydroxy-6-methylflavone with $GABA_A$ receptors expressing the histidine 101 mutations. Lack of receptor expression for an extended period of time due to a number of different factors prevented the completion of affinity studies with this compound, and subsequent comparison with amentoflavone (see Chapters 3 and 4), or inclusion of such results with reviewed affinity studies performed previously in this laboratory (see Discussion), was not possible. Electrophysiological studies of $GABA_A$ receptor function were also affected by methodological challenges. Seasonal variations affecting *Xenopus* oocyte viability and levels of protein expression led to periods where two-electrode voltage clamp electrophysiology experiments did not give consistent or reliable results. In particular, small whole cell current measurements or inconsistent stability measurements precluded the gathering of results. Fortunately, these variations usually disappeared after a time and quality *Xenopus* oocytes displaying reliable and consistent protein expression were available for the majority of experiments. An exception to this, however, was the instance where expression of a particular $GABA_A$ receptor subtype led to inconsistent stability measurements on a permanent basis. This was particularly the case with the $\alpha_1\beta_2$ $GABA_A$ receptor subtype containing the alanine substitution at histidine 101. For unknown reasons, injection of this subtype into *Xenopus* oocytes led to prolonged instability over successive challenges of a constant GABA concentration. This instability could not be resolved over many weeks of experiments, despite the reliable expression of other $GABA_A$ receptor subtypes throughout the same period using the same batches of oocytes, suggesting the instability might be an intrinsic property specific to this particular subtype. Whether or not this is the case, the lack of reliable current measurements with this $GABA_A$ receptor subtype resulted in the inability to examine amentoflavone interaction with the mutant receptor where the γ subunit was not present (see Chapter 3).

Future directions in the context of the preceding studies include a number of different research foci. With regard to flavonoid research, further studies with both natural and synthetic flavonoids are needed to completely elucidate the mechanism of flavonoid interaction at the $GABA_A$ receptor. While comparison of affinity studies with the natural biflavonoid amentoflavone and the synthetic derivative 5′-bromo-2′-hydroxy-6-methylflavone provided some evidence as to the subtype selectivity of flavonoids, more studies are needed to determine whether this conclusion can be extended to the entire class of flavonoid compounds (see Chapters 3 and 4). As well, addition functional studies are required to attain a better understanding of the non-benzodiazepine mechanism of flavonoid interaction at the receptor. Specifically, is the negative modulator activity of amentoflavone due to its actions as an antagonist at the GABA site, or is there a distinct interaction with a novel flavonoid site on the receptor? Additionally, can the non-benzodiazepine interaction of amentoflavone be considered similar to the non-benzodiazepine actions of other naturally occurring monoflavonoids such as apigenin (Goutman et al., 2003), or is the biflavonoid nature of this compound important for its interaction with the $GABA_A$ receptor? Finally, recent findings which report that a synthetic flavonoid, 6-methylflavone, displays non-benzodiazepine site-mediated positive modulator activity at the $GABA_A$ receptor (Hall et al., 2004) necessitates investigation into whether a novel flavonoid site at the $GABA_A$ receptor does indeed exist and whether flavonoids ranging the spectrum of efficacies might interact there. Accordingly, continuing affinity and functional studies with both natural and synthetic flavonoids will be needed to help determine the exact nature of flavonoid actions at the $GABA_A$ receptor.

With respect to further investigations at the benzodiazepine binding site, expanded affinity studies encompassing a range of benzodiazepine site ligands of varying structure and efficacy, will continue to elucidate whether sub sites exist with the benzodiazepine cleft that are associated with ligand structure or efficacy. Further studies with the histidine 101 and serine 205 mutations may help clarify whether these amino acid residues in particular are linked to efficacy and structural properties of benzodiazepine site ligands, respectively (see Discussion). Similar studies with other loop A versus loop C residues may also help differentiate whether these associations are specific to the amino acid residues, or are due to contributions from the larger domains in which these residues are located. Functional studies complementing these types of affinity experiments will also provide a greater understanding of when affinity changes can be expected to be paralleled by efficacy changes, and identify the specific interactions which might predict this tendency.

Further studies with extrasynaptic $GABA_A$ receptor subtypes include those with the chimeric α_6/α_4 recombinant $GABA_A$ receptors where the δ subunit has been incorporated as opposed to the γ subunit (see Chapter 6). Unpublished observations from this laboratory have suggested that incorporation of the δ subunit greatly affects agonist efficacy and potency at $GABA_A$ receptor subtypes as compared to those containing the γ subunit (You, unpublished data). Thus examination of receptors containing the chimeric subunits in combination with a δ subunit may yet reveal specific domains that confer differential sensitivity of α_4- versus α_6-containing GABA$_\text{A}$ receptors to GABA site ligands.

In conclusion, the combination of recombinant expression techniques, chimeric subunits, and site-directed mutagenesis with affinity and functional experiments has provided a powerful means by which to investigate how structure and function are related in the activity of the GABA$_\text{A}$ receptor, and with respect to ligand interaction at the receptor. Ongoing research focus on these types of studies will undoubtedly continue to unravel the complex and diverse mechanisms underlying GABA$_\text{A}$ receptor actions.

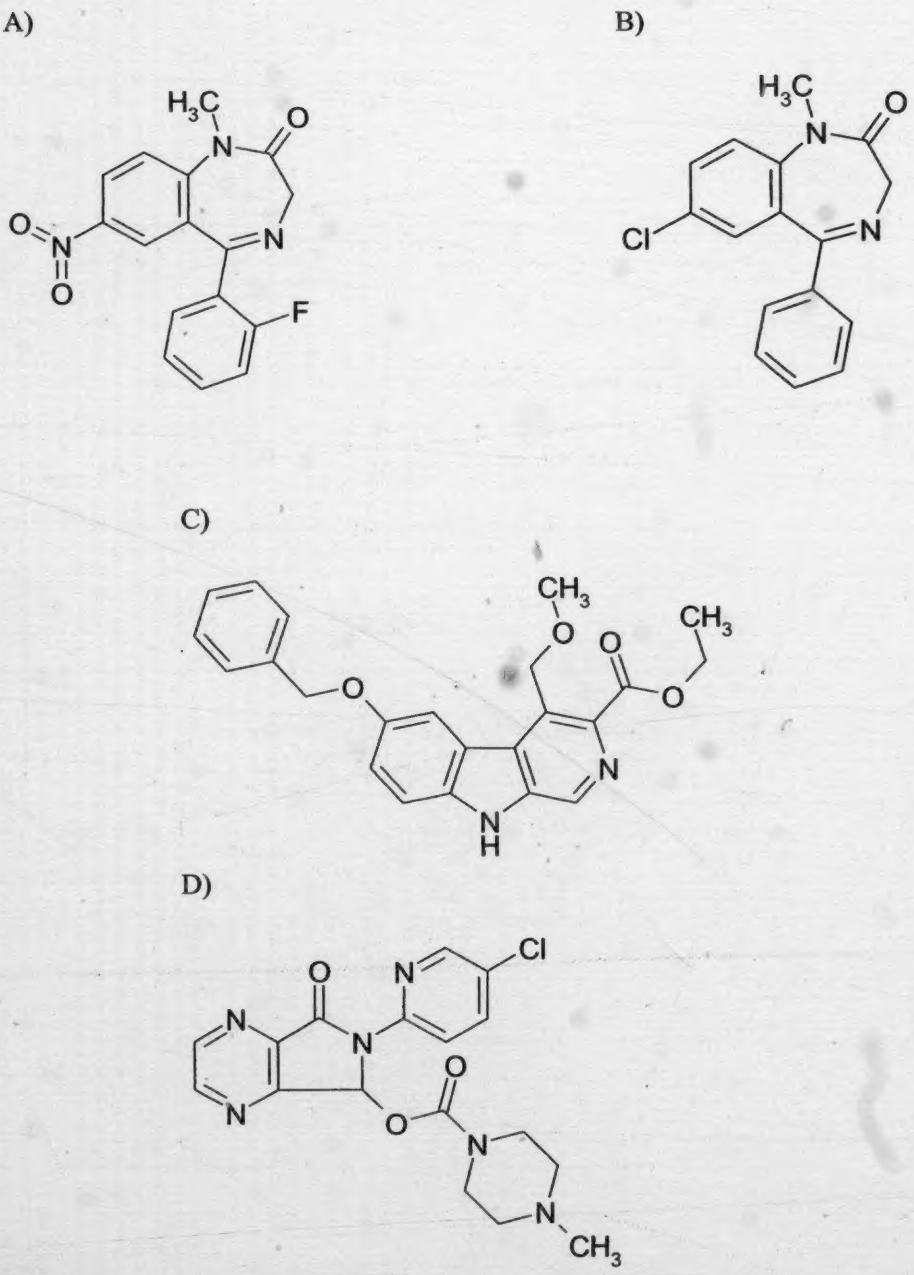

Figure 7-1

Chemical structures of benzodiazepine site positive modulator compounds from affinity studies reviewed in this chapter. The structures of (A) flunitrazepam (FNZ), (B) diazepam (DZ), (C) ZK93423, and (D) zopiclone are depicted for comparison.

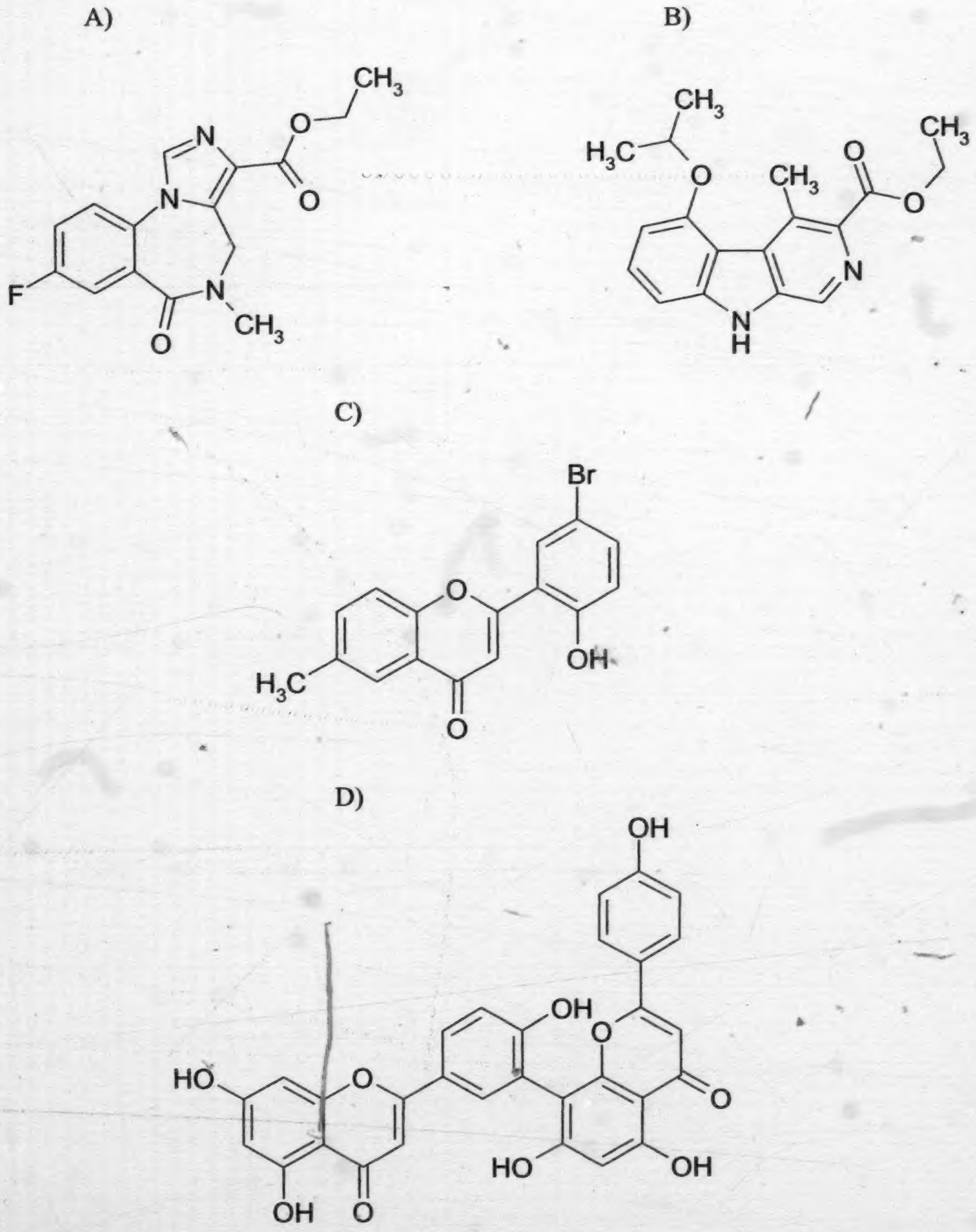

Figure 7-2

Chemical structures of benzodiazepine site antagonist compounds from affinity studies reviewed in this chapter. The structures of (A) Ro15-1788, (B) ZK93426, (C) 5′-bromo-2′-hydroxy-6-methylflavone, and (D) amentoflavone are depicted for comparison.

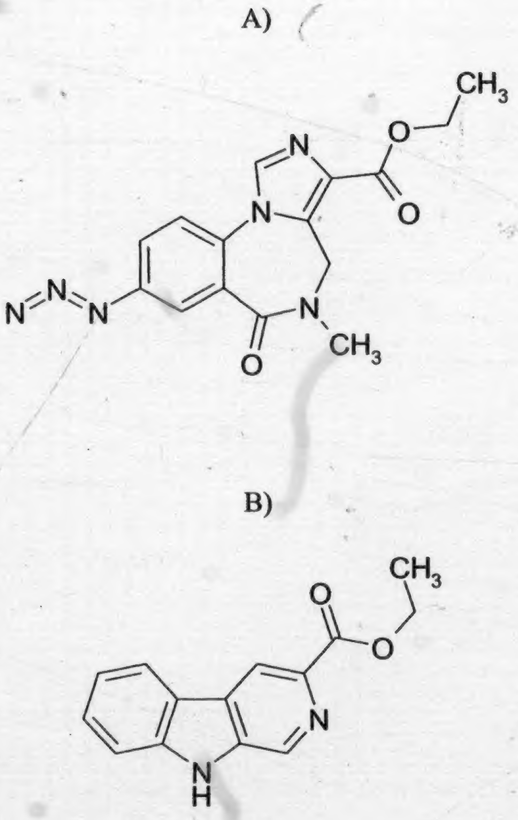

Figure 7-3

Chemical structures of benzodiazepine site negative modulator compounds from affinity studies reviewed in this chapter. The structures of (A) Ro15-4513 and (B) ethyl β-carboline-3-carboxylate (β-CCE) are depicted for comparison.

Affinity (nM)										
	Positive Modulators				Antagonists				Negative Modulators	
	FNZ[a,c]	DZ[b]	ZK [a] 93423	Zopiclone[b]	Ro15-1788 [a,c]	ZK [a] 93426	5'-bromo-2'-[f] hydroxy-6-methylflavone	Amentoflavone [e]	Ro15-4513 [a,c]	βCCE [c,d]
$\alpha_1\beta_2\gamma_2$	6.1	19.4	1.5	51.3	1.3	3.2	7.4	13.8	3.2	0.4
histidine 101 mutation										
phenylalanine	92.8	164	1.9	199	5.0	14.2			0.9	
tyrosine	142	504	4.4	863	90	68.7		30.6	7.0	9.0
lysine	>10 000	>10 000	150	>10 000	26.6	96.0		>10 000	1.5	4.4
glutamate	>10 000	>10 000	130	>10 000	79.1	55.2			2.1	7.2
glutamine	103	1476	8.9	761	8.5	5.3		19.9	0.5	3.3
cysteine	1100				5.3				0.4	
alanine		>10 000		>10 000			>10 000	>10 000		
serine 205 mutation										
asparagine	2.0				25				121	2.8

Table 7-1

Effects of α_1 subunit histidine 101 and serine 205 mutation on the affinity of positive modulator, antagonist, and negative modulator ligand affinity for the benzodiazepine site. [a]Davies et al., 1998; [b]Davies et al., 2000; [c]Derry et al., 2004; [d]Derry, unpublished data; [e]Hansen et al., 2005; [f]Hansen, unpublished data.

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